EVELISE NEVES MACIEL

Este exemplar corresponde à versão final da Tese de Doutorado apresentada ao Curso de Pós-Graduação em Ciências Médicas da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Doutor em Ciências Médicas, Área de Concentração em Ciências Biomédicas do(a) aluno(a) **Evelise Neves Maciel.**
Campinas, 18 de fevereiro de 2004.

Prof(a). Dr(a). Roger Frigério Castilho
Orientador(a)

PERMEABILIDADE MITOCONDRIAL TRANSITÓRIA NA EXCITOTOXICIDADE E NA LESÃO NEURONAL DA ACIDEMIA METILMALÔNICA

CAMPINAS

2004

EVELISE NEVES MACIEL

PERMEABILIDADE MITOCONDRIAL TRANSITÓRIA NA EXCITOTOXICIDADE E NA LESÃO NEURONAL DA ACIDEMIA METILMALÔNICA

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Doutor em Ciências Médicas, área de Ciências Biomédicas.

ORIENTADOR: *PROF. DR. ROGER FRIGÉRIO CASTILHO*
CO-ORIENTADOR: *PROF. DR. ANÍBAL EUGÊNIO VERCESI*

CAMPINAS

2004

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS UNICAMP

Maciel, Evelise Neves

M187p Permeabilidade mitocondrial transitória na excitotoxicidade e na lesão neuronal da acidemia metilmalônica / Evelise Neves Maciel. Campinas, SP : [s.n.], 2004.

Orientadores : Roger Frigério Castilho, Aníbal Eugênio Vercesi
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. *Excitotoxicidade. 2. *Plasticidade neuronal. 3. *Traumatismos cerebrais. 4. *Àcido metilmalônico. 5. Mitocôndria. I. Roger Frigério Castilho. II. Aníbal Eugênio Vercesi. III. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. IV. Título.

Banca examinadora da tese de Doutorado

Orientador: Prof. Dr. Roger Frigério Castilho

Membros:

1. Prof. Dr. Roger Frigério Castilho
2. Prof. Dr. João Batista Teixeira da Rocha
3. Prof. Dr. Sérgio Akira Uyemura
4. Profa. Dra. Elenice Aparecida de Moraes Ferrari
5. Profa. Dra. Fernanda Ramos Gadelha

Curso de pós-graduação em Ciências Médicas da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 18/02/2004

O presente trabalho foi realizado nos Laboratórios de Metabolismo Energético em Neurodegeneração e de Bioenergética, Núcleo de Medicina e Cirurgia Experimental (NMCE), Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), sob a orientação do Professor Dr. Roger F. Castilho, e co-orientação do Professor Dr. Aníbal E. Vercesi, na vigência dos auxílios concedidos pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq) e Fundação de Amparo ao Ensino e Pesquisa da Universidade Estadual de Campinas (FAEP-UNICAMP).

DEDICO

À minha mãe, minha paciência e paz.

Ao meu pai, minha força.

À minha irmã, meu brilho.

Ao Maurício, pelo exemplo de amor e doação incondicional.

Em memória de meus amados avós, Olsen e Davina.

AGRADECIMENTOS

Ao Prof. Dr. Roger Castilho, não somente pela orientação e exemplo de dedicação e estímulo como pesquisador, mas principalmente pela amizade e apoio durante estes anos.

Ao Prof. Dr. Aníbal Vercesi, pela co-orientação deste trabalho, oportunizando o aprendizado junto à sua equipe e pela acolhida carinhosa desde que cheguei em Campinas.

À Dra. Márcia Fagian, não somente pela ajuda cedida na hora da burocracia e técnicas do laboratório, mas principalmente pelo carinho especial.

Às técnicas: Elisangela e Edilene, pela inestimável presteza na preparação de mitocôndrias.

À Adriana, Denise e Natalia, pela demonstração de que a amizade transpõe milhares de quilômetros do Laboratório.

Aos amigos e colegas do Laboratório: Adriana, Alexandre, Anna, Cláudia, Celene, Denise, Endrigo (em memória), Fabiane, Fátima, Félix, Felipe, Francisco, Giovanna, Graziela, Jesus, Jiri, Laura, Leda, Luciane, Márcia, Márcio, Natália, Renata, Ricardo, Solange e Valéria pela descontração, carinho e por terem contribuído para meu desenvolvimento profissional e pessoal.

Às minhas grandes amigas: Alessandra, Graziela, Iva e Thirzá pela força nos momentos difíceis e pelo sorriso nos momentos alegres.

Ao Prof. Dr. João Batista, mestre e amigo, pelo apoio e incentivo nos meus projetos profissionais.

Aos Prof(a)s. Dr(a)s. Moacir Wajner, Diogo Souza, Clóvis Wannmacher, Ângela Wyse, Suzana Wofchuk e Marcus Frizzo pelas discussões e acolhida em seus laboratórios.

Aos Prof(a)s. Dr(a)s. Alicia Kowaltowski e Patrik Brundin, pela colaboração em alguns experimentos.

À Dra. Claúdia Campos, pelas sugestões e discussões enriquecedoras.

À FAPESP, pelo auxílio financeiro, que me permitiu desenvolver um trabalho de qualidade.

Aos funcionários e professores do Curso de Pós-Graduação em Ciências Médicas da UNICAMP.

A DEUS e aos meus guias espirituais, pelo auxílio em todos os momentos, ajudando-me a ter fé e força em todas as situações de minha vida.

A todos que, de alguma forma, contribuíram para a realização deste trabalho.

SUMÁRIO

$\Delta\Psi$	potencial elétrico de membrana
AA	antimicina A
ADP	adenosina 5'-difosfato
AIF	fator indutor de apoptose
AMPA	para-α-amino-3-hidroxil-5-metilsoxanol-proprionato
ANOVA	análise de variância
AQ	ácido quinolínico
ATP	adenosina 5'-trifosfato
BSA	albumina soro bovina
CsA	ciclosporina A
DCF	diclorofluoresceína
DMSO	dimetilsulfóxido
DTT	ditiotreitol
EGTA	etileno glicol-bis(β-aminoetil éter)-*N,N,N',N'*-ácido tetracético
EROs	espécies reativas de oxigênio
FAD	flavina adenina dinucleotídeo
FADH	flavina adenina dinucleotídeo (estado reduzido)
GSH	glutationa reduzida
FCCP	dicarbonil-1,3,6-trifluorofenil-hidrazona
HBSS	solução salina balanceada de Hank
HEPES	ácido 4-(2-hidroxietil)-1-piperazinaetanosulfônico
H_2DCF	diclorodihidrofluoresceína

H_2DCF-DA	diacetado de H_2DCF
MA	malonato
MCR	mitocôndria de cérebro de rato
MFR	mitocôndria de fígado de rato
MMA	metilmalonato
MRR	mitocôndria de rim de rato
NADH	nicotinamida adenina dinucleotídeo (estado reduzido)
NADPH	nicotinamida adenina dinucleotídeo fosfato (estado reduzido)
NAD^+	nicotinamida adenina dinucleotídeo (estado oxidado)
$NADP^+$	nicotinamida adenina dinucleotídeo fosfato (estado oxidado)
NMDA	*N*-methyl-D-aspartato
3-NP	3-nitropropionato
PBS	tampão fosfato salínico
Pi	fosfato inorgânico
PMT	permeabilidade mitocondrial transitória
PPMT	poro de permeabilidade mitocondrial transitória
Safranina	cloreto de 3,7-diamino-2,8-dimetil-5-fenifenazínio
SOD	superóxido dismutase
SDH	succinato desidrogenase
SNC	sistema nervoso central
TBARS	substâncias reativas ao ácido tiobarbitúrico

RESUMO

A permeabilidade mitocondrial transitória (PMT) tem sido implicada na morte celular em diversos modelos de desordens neurodegenerativas, incluindo hipoglicemia, isquemia e trauma cerebral. No presente trabalho, estudamos a PMT em mitocôndrias isoladas de cérebro de rato. A PMT induzida por Ca^{2+} foi estimulada por Na^{+} e prevenida pela combinação de ADP e ciclosporina A (CsA), inibidores da PMT. EGTA, um quelante de Ca^{2+}, ou a inibição da captação de Ca^{2+} por vermelho de rutênio, revertem parcialmente a dissipação do potencial de membrana mitocondrial associada à PMT. Esta foi significantemente inibida por catalase, indicando a participação de espécies reativas de oxigênio (EROs) neste processo. Pela detecção de EROs por meio da oxidação do marcador diclorodihidrofluoresceína (H_2DCF), observamos um aumento de EROs após a captação de Ca^{2+} pela mitocôndria; este aumento de produção de EROs foi estimulado por Na^{+} e totalmente revertido pela adição de ADP e CsA, indicando que a PMT promove estresse oxidativo mitocondrial. Este processo pode ser, em parte, explicado pela depleção de NAD(P)H na indução da PMT induzida por Ca^{2+}, em mitocôndrias de cérebro de rato. Sabe-se que NADPH mantém a função antioxidante dos sistemas glutationa redutase/peroxidase e tioredoxina redutase/peroxidase. Além disto, a PMT está associada com a peroxidação lipídica de membrana. Estes resultados indicam que a PMT, decorrente do aumento de EROs induzido por Ca^{2+} em mitocôndrias isoladas de cérebro, leva a danos oxidativos secundários, como a peroxidação lipídica.

Para estudar o envolvimento da PMT e proteínas Bcl-2 na excitotoxicidade mediada pelo receptor de glutamato *N*-metil-D-aspartado (NMDA), foram realizadas em camundongos transgênicos que hiperexpressam Bcl-2 e em ratos, injeções intra-estriatais de ácido quinolínico, um agonista específico deste receptor. O ácido quinolínico causou uma degeneração de aproximadamente 50% do volume estriatal em ratos e camundongos. Camundongos transgênicos que hiperexpressam a proteína Bcl-2, uma proteína que inibe estímulos apoptóticos, e o tratamento de ratos com CsA não mostraram redução da toxicidade ao ácido quinolínico. Mitocôndrias isoladas de cérebro de ratos tratados com CsA mostraram resistência à indução de permeabilização induzida por Ca^{2+}, indicando proteção contra a PMT. Concluímos que a excitotoxicidade estriatal mediada por ácido quinolínico não é dependente da PMT e da morte celular por apoptose, sensível a Bcl-2.

Mudanças na integridade mitocondrial, liberação de EROs e homeostase de Ca^{2+} estão envolvidas na patogênese de várias desordens neurológicas, incluindo a acidemia metilmalônica e a doença de Huntington (HD). Estas desordens neurodegenerativas cursam com inibição parcial do complexo II da cadeia respiratória mitocondrial. Neste trabalho, nós estudamos os mecanismos pelos quais os inibidores do complexo II da cadeia respiratória, malonato (MA), metilmalonato (MMA) e 3-nitropropionato (3-NP) afetam a função mitocondrial e a sobrevivência neuronal *in vitro*. Observou-se que estes três inibidores, em concentrações que inibem aproximadamente 50% a respiração, induzem permeabilização mitocondrial quando na presença de concentrações micromolares de Ca^{2+}. ADP, CsA e catalase preveniram este efeito, indicando que é mediado por EROs e PMT. Os efeitos de MA, MMA e 3-NP também foram observados em mitocôndrias isoladas de fígado e rim, mas necessitaram uma maior inibição respiratória. Em cérebro, a PMT promovida por inibidores do complexo II foi estimulada pelo aumento da captação/liberação de Ca^{2+} mitocondrial e foi inibida quando a mitocôndria foi pré-carregada com Ca^{2+} ou a entrada de Ca^{2+} na matriz mitocondrial foi somente passiva. Em adição aos experimentos com mitocôndrias isoladas, o efeito de MMA também foi estudado em cultura de células PC12 e fatias de estriado de ratos adultos. MMA promoveu morte celular nas fatias de estriado e cultura de células PC12, num mecanismo sensível à CsA e bongkrekato, e não diretamente relacionado à inibição respiratória. Conclui-se que, em condições em que o complexo II da cadeia respiratória mitocondrial está parcialmente inibida no sistema nervoso central, a morte neuronal envolve a participação da PMT.

ABSTRACT

Mitochondrial permeability transition (MPT) may be involved in several central nervous system disorders including ischemic and hypoglicemic neuronal death. In this study, we show that Ca^{2+}-induced brain mitochondrial PT was stimulated by Na^{+} and totally prevented by the combination of ADP and cyclosporin A (CsA). Removal of Ca^{2+} from the mitochondria suspension by EGTA or inhibition of Ca^{2+} uptake by ruthenium red partially reverted the dissipation of the membrane potential associated with PT. Ca^{2+}-induced brain mitochondrial PT was significantly inhibited by catalase, indicating the participation of reactive oxygen species (ROS) in this process. An increased detection of ROS, measured through dichlorodihydrofluorescein oxidation, was observed after mitochondrial Ca^{2+} uptake. Ca^{2+}-induced dichlorodihydrofluorescein oxidation was enhanced by Na^{+} and prevented by ADP and CsA, indicating that PT enhances mitochondrial oxidative stress. This could be at least in part a consequence of the extensive depletion in NAD(P)H that accompanied Ca^{2+}-induced brain mitochondrial PT. NADPH is known to maintain the antioxidant function of the glutathione reductase/peroxidase and thioredoxin reductase/peroxidase systems. In addition, the occurrence of mitochondrial PT was associated with membrane lipid peroxidation. Our results showed that PT further increases Ca^{2+}-induced oxidative stress in brain mitochondrial leading to secundary damage such as lipid peroxidation.

We studied the participation of PT and the Bcl-2-sensitive apoptotic cell death pathway in glutamate receptor-mediated excitotoxicity. Intrastriatal infusions of the *N*-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid caused massive striatal neurodegeneration in both rats and mice. Interestingly, transgenic mice overexpressing human Bcl-2 and rats systemically treated with CsA did not exhibit reduced sensitivity to quinolinic acid-induced striatal toxicity. In addition, isolated brain mitochondrial from CsA-treated rats showed resistance to Ca^{2+}-induced dissipation of the membrane potential, indicating protection against PT. We conclude that quinolinic acid-mediated striatal excitotoxicity is not dependent on PT and Bcl-2-sensitive apoptotic cell death pathways.

Changes in mitochondrial integrity, reactive oxygen species release and Ca^{2+} handling are proposed to be involved in the pathogenesis of many neurological disorders including methylmalonic acidemia and Huntington's disease (HD), which exhibit partial mitochondrial complex II inhibition. We studied the mechanisms by which the respiratory

chain complex II inhibitors malonate (MA), methyl-malonate (MMA) and 3-nitropropionate (3-NP) affect mitochondrial function and neuronal survival. We observed that all three inhibitors, at concentrations which inhibit respiration by 50%, induced mitochondrial permeabilization when in the presence of low Ca^{2+} concentrations. ADP, CsA and catalase prevented this effect, indicating it is mediated by ROS and PT. The effects of MA, MMA and 3-NP were also present in mitochondria isolated from liver and kidney, but required more significant respiratory inhibition. In brain, PT promoted by complex II inhibitors was stimulated by increasing Ca^{2+} cycling and absent when mitochondria were pre-loaded with Ca^{2+} or when only passive Ca^{2+} uptake was present. In addition to the experiments on isolated mitochondria, we determined the effect of MMA on cultured neural cells model and freshly prepared brain slices. MMA promoted cell death in striatal slices and PC12 cells, in a manner attenuated by CsA and bongkrekate and unrelated to direct respiratory inhibition. We propose that under conditions in which complex II is partially inhibited in the central nervous system, neuronal cell death involves the induction of M PT.

1- INTRODUÇÃO

1.1-ISQUEMIA CEREBRAL

Devido ao grande potencial de morbidade e mortalidade, as lesões no sistema nervoso central (SNC) causam sério impacto sócioeconômico. Os fatores etiológicos passíveis de causar lesões neurológicas são vários, entre eles: hipertensão arterial, doenças vasculares, infecções, traumatismo crânio-encefálico e doenças hereditárias. Na maioria das vezes, as lesões no SNC deixam seqüelas, como déficits motores, cognitivos ou de memória, dependendo do local atingido e/ou do tamanho da lesão (PULSINELLI, 1992; LIPTON, 1999).

Dentre as lesões no SNC, podem citar os acidentes cerebrovasculares cerebrais (AVC). Os AVC podem ser divididos em duas principais categorias: hemorrágicos ou isquêmicos. Nos acidentes vasculares hemorrágicos existem hemorragias locais, com outros fatores complicadores, tais como aumento da pressão intracraniana, edema cerebral, entre outros, levando a sinais nem sempre focais. Os acidentes isquêmicos são os mais comuns, cerca de 80%, e ocorrem devido à falta ou redução do fluxo sangüíneo para os tecidos, resultando em privação de oxigênio e glicose, além de diminuição na síntese de alguns produtos metabólicos produzidos pelo cérebro (SIESJO et al., 1974; PULSINELLI, 1992; LIPTON, 1999).

A gravidade da lesão isquêmica é decorrente do grau e do tempo de duração da isquemia, podendo ser classificada temporalmente em transitória ou perene e, topograficamente, em global, focal ou multifocal; além de total ou parcial, nos modelos que se referem à redução no fluxo sangüíneo (GINSBERG e BUSTO, 1989; HUNTER et al., 1995; GINSBERG, 1997). Os modelos de isquemia transitória ou de curta duração (< 15 min em ratos) apresentam um período de reperfusão, ou seja, aqueles em que o sangue torna a fluir nos vasos sangüíneos e a homeostase iônica do tecido é rapidamente restaurada (15-30 min) (SIEMKOWICZ, 1981; PULSINELLI e DUFFY, 1983; LIPTON, 1999), enquanto que nas isquemias perenes ou de longa duração (1-2 h), a homeostase iônica do tecido é parcialmente restaurada após a reperfusão e são caracterizadas pela morte de células neuronais e gliais entre 36-72 h após o insulto (LINN et al., 1987; PULSINELLI, 1992; LIPTON, 1999).

A isquemia cerebral global pode afetar o cérebro inteiro, podendo induzir a perda da pressão sangüínea e aumento da pressão intracraniana, levando a edemas cerebrais. A isquemia focal afeta partes do cérebro, podendo causar danos permanentes em neurônios específicos e regiões particulares do SNC, sugerindo uma vulnerabilidade seletiva que ocorre em áreas menos vascularizadas (SCHMIDT-KASTNER e FREUND, 1991; LIPTON, 1999). Vários pesquisadores têm demonstrado que os neurônios diferem em sua sensibilidade ao dano isquêmico e em sua capacidade de recuperação após o insulto (PULSINELLI, 1992; 1997; AUER, 1998). Algumas áreas apresentam maior vulnerabilidade, particularmente a região CA1 do hipocampo, onde se observa morte de quase todos os neurônios após uma breve interrupção do fluxo sangüíneo cerebral (PULSINELLI, 1992; AUER, 1998). Depois das células piramidais da região CA1 do hipocampo, as células mais sensíveis à isquemia são os neurônios das 3.ª e 4.ª camadas corticais, estriado, septo, CA3, tálamo-óptico e substância nigra (SCHIMIDT-KASTNER e FREUND, 1991; PULSINELLI, 1992; KOGURE e KATO, 1993). Em contraste, as células granulares do giro denteado e astrócitos possuem uma alta resistência a insultos isquêmicos (KIRINO, 1982; PULSINELLI, 1992). A susceptibilidade do sistema nervoso central à isquemia é multifatorial e ocorre principalmente pela convergência das propriedades bioquímicas de alguns grupos celulares, além da severidade do insulto e da região anatômica na qual estas células se encontram (PULSINELLI, 1997; AUER, 1998).

Hirschenerg e Winterstein (1917) sugeriram pela primeira vez, utilizando modelos de preparações de medula espinhal de rãs, o consumo de glicose pelo SNC. Em 1924, neste mesmo modelo, observaram a produção de ácido lático na ausência de oxigênio e, finalmente em 1951, Himwich demonstrou o consumo de oxigênio e glicose contínuo pelo SNC, resultante da aferição dos níveis arterial e venoso (DIXON, 1965). Uma vez determinado que o metabolismo do SNC depende do constante aporte de oxigênio e glicose postulou-se que a morte das células ocorria simplesmente por "falência energética" (KOGURE e KATO, 1993). De fato, o tecido cerebral possui apenas uma pequena reserva de energia e uma alta taxa metabólica sendo, assim, sensível à hipóxia-isquêmica. Este pequeno estoque de energia é rapidamente consumido quando o aporte sistêmico falha, desencadeando uma série de alterações metabólicas que levam à morte celular (SCHIMIDT-KASTNER e FREUND, 1991; DUGAN e CHOI, 1999; LIPTON, 1999).

As alterações metabólicas e iônicas na região do insulto não são homogêneas. No centro ou core, onde o fluxo sangüíneo é 20% menor que o normal, as células sofrem uma despolarização após o início da isquemia e rapidamente morrem. Entre o core e regiões não afetadas do cérebro, encontra-se a penumbra, uma área com fluxo sangüíneo limitado, porém com parte da energia metabólica preservada. Na penumbra, as células podem despolarizar em resposta aos níveis elevados de glutamato e K^+. Repetidas despolarizações nestas células são chamadas de despolarizações perinfartos e podem manter-se por até 8 h após o infarto. Portanto, o tamanho da lesão tecidual dependerá do número e intensidade das despolarizações perinfartos. O uso de drogas que diminuem o número de despolarizações, diminuem o tamanho do insulto (DIRNAGI et al., 1999).

Durante a isquemia e reperfusão ocorre uma liberação de aminoácidos excitatórios, em especial o glutamato, um influxo de Ca^{2+} e um aumento de radicais livres, marcando a fase denominada excitotóxica (LIPTON, 1999). O conceito de excitotoxicidade foi elaborado a partir de evidências provindas de modelos experimentais para isquemia. A primeira delas foi a constatação de um aumento nos níveis extracelulares de glutamato durante a isquemia e a segunda, o fato de que o bloqueio de receptores NMDA inibe os fenômenos decorrentes da descarga glutamatérgica (SCHIMIDT-KASTNER e FREUND, 1991). Receptores metabotrópicos glutamatérgicos tipo 1 (mGlu1) parecem também exercer um papel importante na morte celular pós-isquêmica, uma vez que a administração de antagonistas específicos destes receptores atenua o dano neuronal induzido pela isquemia (PELLEGRINI-GIAMPIETRO et al., 1999).

1.2-ACIDEMIAS ORGÂNICAS

1.2.1-Definição

Acidemias orgânicas são doenças hereditárias autossômicas recessivas, caracterizadas pelo distúrbio no metabolismo de aminoácidos, glicídios ou lipídeos, causando acúmulo tecidual de um ou mais ácidos carboxílicos (CHALMERS e LAWSON, 1982; SCRIVER et al., 2001). Os defeitos de oxidação dos ácidos graxos são usualmente devido à deficiência severa da atividade de uma enzima, geralmente do metabolismo de aminoácidos, podendo também comprometer o metabolismo de lipídeos ou carboidratos.

Recentemente utilizou-se o termo acidemias orgânicas cerebrais para distúrbios do metabolismo dos ácidos orgânicos com manifestações neurológicas (HOFFMANN et al., 1993; HOFFMANN, 1994). As manifestações clínicas normalmente ocorrem na primeira semana de vida e os sintomas predominantes são: encefalopatia aguda ou crônica, com letargia, hipotonia, retardo mental, atrofia cerebral, apatia, episódios epiléticos, isquemia cerebral, coma e convulsões (LEHNERT et al., 1994; FENTON et al., 2001; SWEETMAN e WILLIAMS, 2001).

1.2.2-Aspectos Bioquímicos

Os ácidos graxos, armazenados em adipócitos na forma de triglicerídeos, constituem importante fonte de energia para a maioria dos tecidos. A oxidação dos ácidos graxos passa necessariamente pela produção de acetil coenzima A (acetil-CoA) pela mitocôndria num processo conhecido como β-oxidação de ácidos graxos. Cada ciclo da β-oxidação produz um NADH, um $FADH_2$ e uma acetil-CoA. O acetil, transportado por CoA, é oxidado pelo Ciclo do Ácido Cítrico gerando um $FADH_2$ e três NADHs adicionais, os quais são reoxidados por meio da fosforilação oxidativa, formando ATP. A acetil-CoA, além de ATP, produz CO_2 e H_2O nos tecidos periféricos ou saem da mitocôndria sob a forma de citrato para a biossíntese do colesterol (WANNMACHER e DUTRA, 1988; MARKS et al., 1996).

A maioria dos ácidos graxos possui um número par de carbonos, convertidos por completo em acetil-CoA; entretanto, uma pequena porção de ácidos graxos contém um número ímpar de átomos de carbono. Estes ácidos graxos são metabolizados pela via da β-oxidação; contudo, a clivagem tiolítica final fornece acetil-CoA e propionil-CoA, a qual é convertida em succinil-CoA para entrar no Ciclo do Ácido Cítrico. O propionato e a proprionil-CoA são também produzidos pela oxidação dos aminoácidos isoleucina, valina e metionina e do metabolismo das bases pirimídicas timina e uracila (WANNMACHER e DUTRA, 1988; MARKS et al., 1996).

Nos hepatócitos, durante períodos de jejum e até mesmo durante o exercício prolongado, acetil-CoA é convertida em corpos cetônicos, que são liberados na corrente sangüínea e servem como fonte de energia para o tecido muscular e rins. Além disto, em

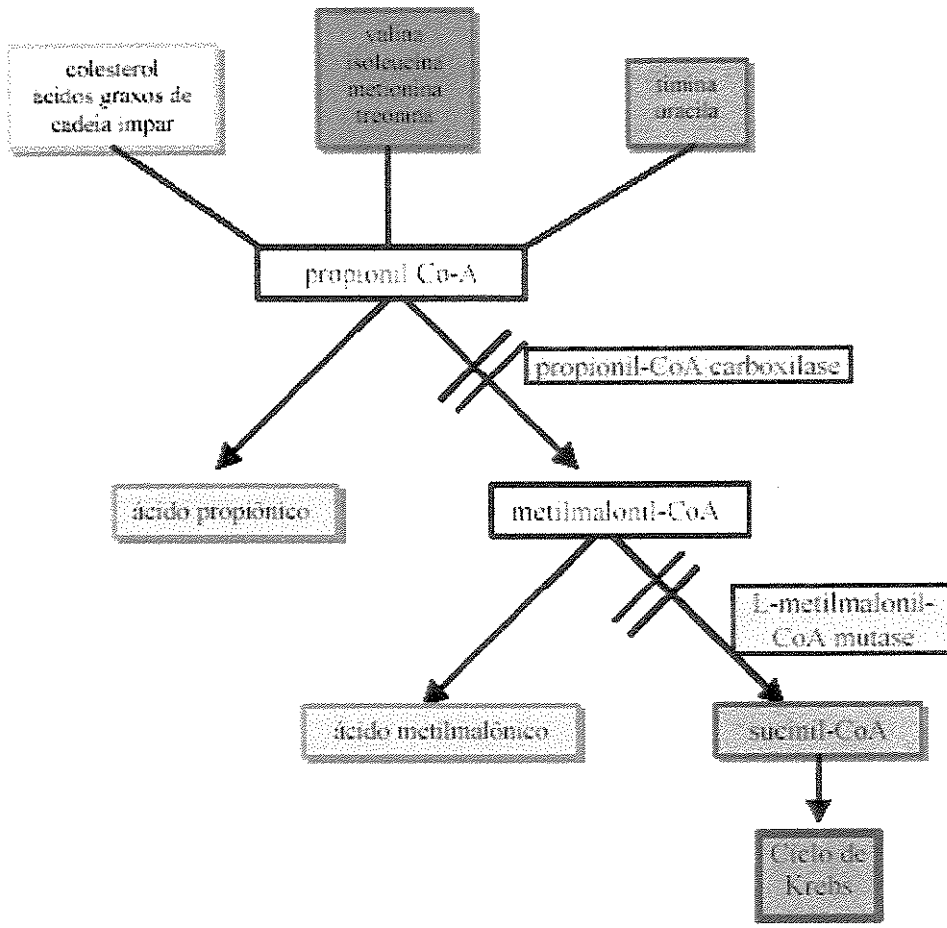
situações de grande deficiência de energia, os corpos cetônicos são utilizados como fonte de energia pelo cérebro. Através da corrente sangüínea, os corpos cetônicos, entram no cérebro, diminuindo assim a necessidade de utilização de aminoácidos das proteínas musculares para produção de glicose (gliconeogênese) (WANNMACHER e DUTRA, 1988; MARKS et al., 1996).

1.2.3-Acidemias Propiônica e Metilmalônica

As acidemias propiônicas e metilmalônicas estão entre as acidemias orgânicas mais freqüentes com incidência de 1:40.000 e 1:48.000, de nascidos vivos, para as acidemias propriônica (DURAN et al., 1994) e metilmalônica (SCRIVER et al., 2001) respectivamente, e são caracterizadas por severa acidose metabólica, hiperglicemia e hiperamonemia.

As acidemias propiônica e metilmalônica caracterizam-se pelo acúmulo predominante dos ácidos propiônico (PA) e metilmalônico (MMA), devido à deficiência nas enzimas L-propionil-CoA carboxilase e L-metilmalonil-CoA mutase, respectivamente (Esquema 1).

Alterações no metabolismo energético, causadas pelo ácido propiônico incluem a inibição de importantes enzimas envolvidas na produção de energia, tais como: citrato sintase (EVANGELIOU et al., 1985), complexo piruvato desidrogenase (GREGERSEN, 1981), além de enzimas da oxidação de ácidos graxos, reduzindo assim a atividade do Ciclo do Ácido Cítrico (GLASGOW e CHASE, 1976; ESFANDIARI et al., 1997) e, conseqüentemente, a síntese de ATP (ROE et al., 1983).



ESQUEMA 1-Rota metabólica dos ácidos propiônico e metilmalônico (Fonte: PETTENUZZO, 2001).

O acúmulo de MMA é tóxico para células de vários tecidos, incluindo neurônios e células gliais. Estudos sobre estes efeitos tóxicos sugerem uma inibição competitiva da succinato desidrogenase, além de aumento de lactato e redução na produção de CO_2 no cérebro de ratos jovens (WAJNER et al., 1992; OKUN et al., 2002), decorrentes da redução na produção de energia cerebral, bem como de alterações na fosforilação de proteínas cerebrais. Estudos recentes, em cultura de células de córtex e estriado de ratos, mostraram uma viabilidade neuronal em torno de 10% durante a incubação com 10 mM de MMA, além de uma diminuição na relação ATP/ADP após o tratamento das células com MMA; estes efeitos podem ser explicados por meio da inibição da cadeia respiratória

mitocondrial (McLAUGHLIN et al., 1998). Deste modo, a acidemia metil-malônica provoca uma "isquemia metabólica", dificultando o transporte de elétrons mitocondrial. Em crianças com acidose metilmalônica, o dano cerebral pode se iniciar logo após o início da ingestão de proteínas, e apresenta piora gradativa, resultando geralmente em manifestações neurológicas nos primeiros meses de vida (SWEETMAN e WILLIAMS, 2001).

1.3-ISQUEMIA CEREBRAL E EXCITOTOXICIDADE

1.3.1-Receptor *N*-Metil-D-Aspartato (NMDA)

O glutamato é o principal neurotransmissor excitatório do SNC de mamíferos. Os receptores glutamatérgicos são proteínas de membrana que medeiam a transmissão excitatória na superfície celular por meio da ligação do glutamato (HOLLMANN e HEINEMANN,1994; NAKANISHI e MASU, 1994) e são classificados em ionotrópicos (*N*-metil-D-aspartato ou NMDA, AMPA e kainato) e metabotrópicos.

Os receptores metabotrópicos (grupos I e II) estão associados às proteínas G e somente permitem a entrada de íons monovalentes, enquanto os receptores ionotrópicos NMDA, AMPA e kainato possibilitam a entrada de íons Ca^{2+} e Na^{+} (HOLLMANN e HEINEMANN, 1994; WESTBROOK, G.L., 1994) (Figura 1).

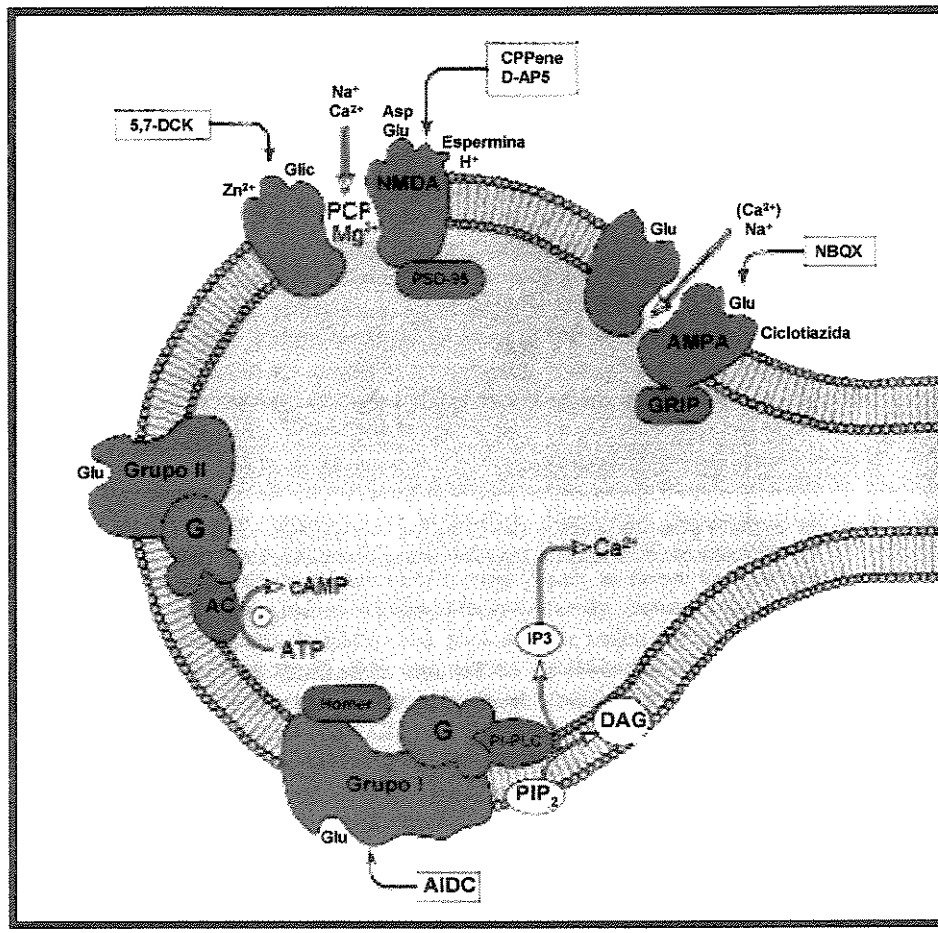


FIGURA 1-Representação dos receptores ionotrópicos (AMPA e NMDA) e metabotrópicos (grupos I e II) de glutamato no terminal sináptico (Adaptado de: DINGLEDINE e MCBAIN In: Siegel, G. J.; (ed.): "Basic Neurochemistry", 1999). Abreviações: PIP_2: fosfatidil inositol-4,5-bifosfato; IP_3: inositol-1,4,5-trifosfato; DAG: diacilglicerol; PSD-95, GRIP e Homer: proteínas citoplasmáticas; 5,7-DCK: ácido 5,7-dicloroquinurênico; CPPene: ácido 3-(2-carboxipiperazina-4-il) 1-propenil-1-fosfórico; DAP5: ácido D-2-amino-5-fosfopentanóico; NBQX: 6-nitro-7-sufanobenzol(f) quinoxalina-2-,3-diona; Glic: glicina; PCP: fenilciclidina; cAMP: adenosina monofosfato cíclico; AC: adenilato ciclase; AIDC: 1-dicarboxilato (antagonista competitivo mGLuR1); PI-PLC: fosfolipase C específica a fosfaditil inositol.

A atividade do receptor NMDA é regulada por sítios alostérios:

- sítio para agonistas, como glutamato e ácido quinolínico e antagonistas competitivos;
- sítio para a glicina;
- sítio para poliaminas;
- sítio PCP (anestésicos) e MK-801 (antagonista à resposta do receptor);
- sítio para Mg^{2+}, bloqueia o canal de maneira voltagem-dependente;
- sítio para Zn^{2+}, bloqueia o canal de maneira voltagem-independente;
- sítio redox, sensível ao poder de oxi-redução dos neurônios;
- sítio sensível a prótons.

O receptor NMDA auxilia a transmissão sináptica, por meio do influxo de Ca^{2+} e Na^{+} e efluxo de K^{+}, além de contribuir para a plasticidade neuronal. Entretanto, tem-se demonstrado que o receptor NMDA é a principal via de excitotoxicidade na morte neuronal, provavelmente pela alta permeabilidade a íons Ca^{2+} (CHOI, 1988; REYNOLDS, 1998), sugerindo a participação de receptores NMDA na lesão neuronal após isquemia/reperfusão e em diversas desordens neurodegenerativas, incluindo doenças de Parkinson, Alzheimer e Huntington (CHOI, 1988; MELDRUM e GARTHWAITE, 1990; LINPTON e ROSENBERG, 1994; LANCELOT e BEAL, 1998).

1.3.2-Excitotoxicidade

Durante a isquemia cerebral, a concomitante redução da fosforilação oxidativa (produção de ATP) mitocondrial e a falta de substrato para a glicólise levam a um rápido declínio do ATP tecidual (LJUNGGREN et al., 1974; NORDSTROM e SIESJO, 1978; LIPTON, 1999) e há um acúmulo de lactato, tornando a célula acidótica (HACKEL e JENNINGS, 1988). Como conseqüência da redução da razão ATP/ADP, o gradiente iônico ao longo da membrana plasmática não pode ser mantido, ocorrendo despolarização da membrana plasmática, efluxo de K^{+} para o meio extracelular, influxo de Ca^{2+} e Na^{+} para o

citosol acompanhado de influxo de Cl^- e água, resultando em inchamento celular (LIPTON, 1999).

Paralelamente a este aumento de despolarização ao longo da membrana plasmática, ocorre uma massiva liberação de neurotransmissores, como glutamato, para o meio extracelular. Uma vez no meio extracelular, o glutamato poderá estimular receptores NMDA da membrana plasmática de neurônios que promoverão maior influxo de Ca^{2+} e Na^+ do meio extracelular para o citosol (LIPTON, 1999). Esta toxicidade pode ser exacerbada pela diminuição dos estoques de energia intracelular (ALBIN e GREENAMYRE, 1992; DUGAN e CHOI, 1999). O glutamato também causa aumento de Ca^{2+} intracelular por ativação de receptores para-α-amino-3-hidroxil-5-metilsoxanol-proprionato (AMPA) e por levar indiretamente à ativação de canais de Ca^{2+} voltagem-dependentes (FAROOQUI et al, 1995).

Vários fatores são relacionados à morte neuronal durante a isquemia e reperfusão, como ativação de proteases, fosfolipases e calcineurina (NAGAHIRO et al., 1998), que desencadeiam uma série de reações levando à morte celular (LIPTON, 1999). Tem-se demonstrado que o aumento de Ca^{2+} intramitocondrial tem um importante papel na morte neuronal em modelos de isquemia e reperfusão (CASTILHO et al., 1998, 1999; FISKUM et al., 1999) por desencadear a produção de espécies reativas de oxigênio (EROs) pela cadeia respiratória mitocondrial ou outros sistemas produtores de EROs (COYLE e PUTTFARCKEN, 1993; DAWSON et al., 1993; CASTILHO et al., 1999), promovendo danos ao citoesqueleto e à membrana plasmática (DUGAN e CHOI, 1999). Esta cascata de eventos que antecedem a morte neuronal, denominamos excitotoxicidade. A Figura 2 resume os efeitos de um evento isquêmico relacionados à homeostase iônica intracelular.

1.3.3-Ácido Quinolínico

O ácido quinolínico (ácido piridina-2,3-dicarboxílico) (AQ) é um metabólito participante da rota de biossíntese de nicotinamida adenina dinucleotídeo (NAD^+) (NISHIZUKA e HAYAISHI, 1963; GHOLSON et al., 1964). Apesar da importância na biossíntese de NAD oxidado, o AQ é encontrado em baixas concentrações no cérebro e, utilizado como modelo de excitotoxicidade em lesões estriatais (BEAL et al, 1986).

Efeitos excitotóxicos do AQ parecem estar relacionados à ativação de receptores NMDA (agonista glutamatérgico), causando um maciço influxo de Ca^{2+} nos neurônios, desencadeando mecanismos neurotóxicos, incluindo produção de EROs (LAFON-CAZAL et al, 1993). A participação de receptores NMDA na neurotoxicidade do AQ é constatada pelo fato do MK-801, um antagonista seletivo de receptores NMDA, reverter o efeito de AQ (BEAL et al., 1988; QIN et al., 1996). A infusão intraestriatal de AQ tem sido utilizada como um modelo experimental para o estudo de doenças neurodegenerativas, como a doença de Huntington (BEAL et al, 1986).

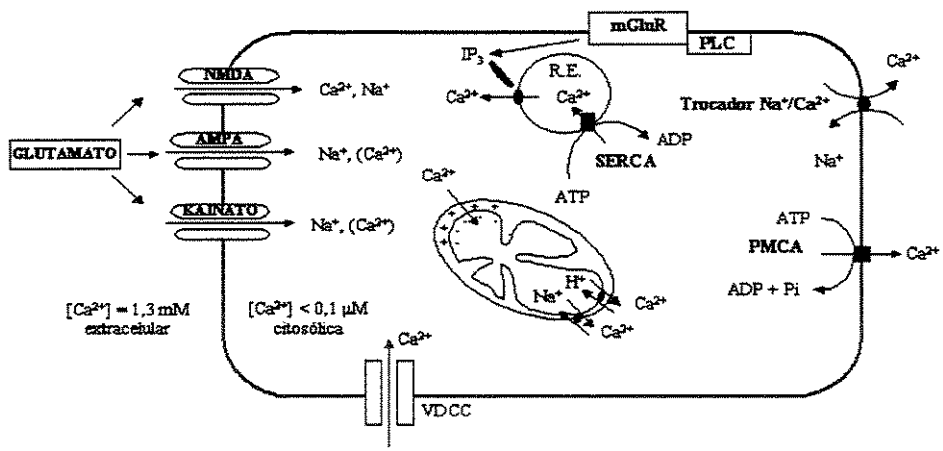


FIGURA 2-Mecanismos relacionados à homeostase iônica intracelular numa célula neuronal. O aumento da concentração de glutamato no meio extracelular resulta na ativação de receptores metabotrópicos (mGluR) que irá levar à mobilização de Ca^{2+} do retículo endoplasmático (RE) via inositol tri-fosfato (IP3). Ao mesmo tempo, a ativação de receptores ionotrópicos de glutamato (AMPA e Kainato) promove o influxo de Na^{+} para o citoplasma, enquanto a ativação do receptor ionotrópico NMDA promove o influxo de Ca^{2+} e Na^{+}. A ativação do canal de Ca^{2+} voltagem-dependente (VDCC) também pode contribuir para o acúmulo de Ca^{2+} no citosol. A homeostase da concentração de Ca^{2+} no citosol será mantida por meio da extrusão deste cátion para o meio extracelular realizada por um trocador Ca^{2+}/Na^{+} e uma Ca^{2+}-ATPase da membrana plasmática (PMCA). Dentro da célula, o Ca^{2+} em excesso poderá ser acumulado pelo RE por meio da Ca^{2+}-ATPase e pela mitocôndria.

1.4-HOMEOSTASE INTRACELULAR DE Ca^{2+}

O íon cálcio é um importante mensageiro secundário em várias células, incluindo neurônios. O Ca^{2+} transmite sinais extracelulares, que regulam o metabolismo mitocondrial, a síntese protéica, a fosforilação e a expressão gênica (CARAFOLI, 1987; CLAPHAM, 1995). Nos neurônios a concentração de Ca^{2+} livre no citosol é de aproximadamente 0,1 μM, mantida cerca de 10.000 vezes abaixo da concentração extracelular (aproximadamente 1,3 mM) (CARAFOLI, 1987; CLAPHAM, 1995). Este alto gradiente eletroquímico de Ca^{2+} entre os compartimentos intra e extracelulares é essencial para a sua função como carregador de sinais bioquímicos para o interior das células. A distribuição do Ca^{2+} intracelular é controlada por processos de transporte do íon ao longo da membrana plasmática e das membranas de organelas subcelulares, como o retículo endoplasmático, núcleo e mitocôndria (GUNTER e GUNTER, 1994; ORRENIUS et al., 2003).

No retículo endoplasmático a entrada de Ca^{2+} é catalisada pela Ca^{2+}-ATPase, denominada SERCA, que transloca 2 Ca^{2+} para cada ATP hidrolisado (DE MEIS e VIANNA, 1979). O efluxo de Ca^{2+} se dá por um canal estimulado por inositol trifosfato (IP_3) (BERRIDGE, 1993) e cafeína (através do receptor de rianodina) (SORRENTINO e VOLPE, 1993). A alta afinidade da Ca^{2+}-ATPase com o cátion confere ao retículo um importante papel na regulação da concentração de Ca^{2+} livre no citosol.

O influxo de Ca^{2+} mitocondrial ocorre por um processo eletroforético, em resposta ao potencial elétrico gerado pela membrana interna (negativo internamente), devido à atividade da cadeia respiratória ou hidrólise de ATP (GUNTER e GUNTER, 1994).

Na mitocôndria, existem dois mecanismos de efluxo de íons Ca^{2+}: Na^+ dependente, ocorre por intermédio do antiporter Na^+/Ca^{2+} (troca Ca^{2+} por Na^+); e, Na^+ independente, ocorre por intermédio do antiporter H^+/Ca^{2+} (troca Ca^{2+} por H^+). A principal função do Ca^{2+} na matriz mitocondrial é, provavelmente, o estímulo da atividade de enzimas regulatórias do Ciclo do Ácido Cítrico (desidrogenases cetoglutárica e isocítrica).

Estudos de TYMIANSKI et al., 1993 evidenciaram que o influxo de Ca^{2+} por meio de canais dependentes de voltagem não apresenta toxicidade aos neurônios, porém o influxo de Ca^{2+} através de receptores NMDA resulta em morte neuronal.

1.5-CADEIA RESPIRATÓRIA MITOCONDRIAL E PRODUÇÃO DE ESPÉCIES REATIVAS DE OXIGÊNIO (EROs)

A mitocôndria é responsável pela síntese de ATP, através da fosforilação oxidativa (HATEFI, 1995), além de estar envolvida na captação e liberação de Ca^{2+}, na geração de espécies reativas de oxigênio e na permeabilidade mitocondrial transitória (PMT) (KOWALTOWSKI et al., 2001).

A energia necessária para o processo de fosforilação provém do potencial eletroquímico de prótons gerado pela cadeia de transporte de elétrons que reduz o O_2 à H_2O. Este consumo O_2 pelas mitocôndrias é conhecido como respiração mitocondrial (Figura 3). Os elétrons provenientes das coenzimas NADH e $FADH_2$ reduzidas durante a oxidação de carboidratos, aminoácidos e ácidos graxos, são transferidos à NADH desidrogenase (Complexo I) ou para succinato desidrogenase ligada à FAD (Complexo II). A partir dos complexos I e II, os elétrons são transferidos para a ubiquinona (UQ), citocromo *c* (Complexo III) e finalmente para o O_2 (Complexo IV) com formação de H_2O (LEHNINGER, 1997; VOET, 2002).

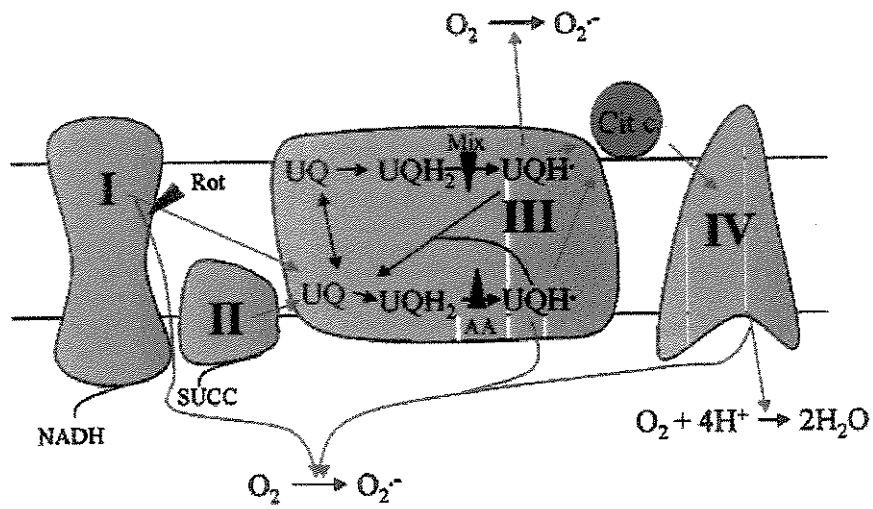


FIGURA 3-Cadeia Respiratória Mitocondrial e Produção de EROs (Fonte: KOWALTOWSKI e VERCESI, 2001).

Apesar de o funcionamento da cadeia de transporte elétrons mitocondrial ter um importante papel na síntese de ATP, por meio do potencial eletroquímico de prótons gerado, a cadeia respiratória mitocondrial produz constantemente EROs (Figura 3). O radical superóxido ($O_2^{\cdot-}$) é gerado principalmente pela NADH desidrogenase (Complexo I) e pela coenzima Q/complexo III (BOVERIS e CHANCE, 1973; TURRENS e BOVERIS, 1980). A geração de $O_2^{\cdot-}$, ao nível do complexo I, pode ser promovida pela presença de substratos respiratórios que geram NADH, como o malato, glutamato e piruvato (TURRENS e BOVERIS, 1980), e estimulada por rotenona, um inibidor da transferência de elétrons do complexo I. A coenzima Q também estimula sensivelmente a geração de $O_2^{\cdot-}$ pela NADH desidrogenase (TURRENS, 1997), provavelmente durante a doação de elétrons do ânion semiquinona, que é um radical livre para o oxigênio molecular. O vazamento de elétrons, ao nível da coenzima Q/complexo III, é estimulado por succinato, cianeto e antimicina A (BOVERIS et al., 1976, TURRENS, 1997, KOWALTOWSKI et al., 1998). A antimicina A tem um efeito estimulatório, pois promove o acúmulo de radicais semiquinona, formados na face citosólica da membrana mitocondrial interna. Mixotiazol, um inibidor da formação de radicais semiquinona, na face citosólica da membrana interna mitocondrial, previne a geração de radical $O_2^{\cdot-}$ ao nível do complexo III (TURRENS, 1997;

KOWALTOWSKI et al., 1998). Em condições normais, o $O_2^{\cdot-}$ é transformado em peróxido de hidrogênio (H_2O_2) na matriz mitocondrial pela ação da superóxido dismutase dependente de manganês (MnSOD). O H_2O_2 é reduzido a H_2O pela glutationa reduzida (GSH), a qual é mantida neste estado pelo NADPH. Assim, a célula possui um sistema antioxidante eficiente composto por GSH, SOD, NADPH, glutationa peroxidase (GPx) e glutationa redutase (GRd), além de vitaminas C e E (KOWALTOWSKI e VERCESI, 1999; GUTTERIDGE e HALLIWELL, 2000).

O Ca^{2+} parece ser o principal agente estimulador da geração de EROs (KOWALTOWSKI e VERCESI, 1999). O Ca^{2+} intramitocondrial liga-se a cardiolipinas, que possuem cabeça polar eletronegativa, na face interna da membrana mitocondrial interna causando alterações ultra-estruturais da cadeia respiratória mitocondrial, que facilita a produção de $O_2^{\cdot-}$ e, conseqüentemente, de H_2O_2 (GRIJALBA et al., 1999). As cardiolipinas possuem cabeça polar eletronegativa e estão presentes, em altas concentrações, na membrana mitocondrial interna. O H_2O_2 pode ligar-se ao Fe^{2+} na matriz mitocondrial, estimulando a reação de Fenton e a produção do radical hidroxil que ataca tióis de proteínas, lipídeos e DNA mitocondrial (CASTILHO et al., 1995; VERCESI et al., 1997).

As EROs têm sido sugeridas em muitos processos degenerativos, incluindo envelhecimento celular e morte celular por apoptose e necrose (GREEN e REED, 1998; KOWALTOWSKI e VERCESI, 1999; ORRENIUS et al., 2003).

1.6-PERMEABILIDADE MITOCONDRIAL TRANSITÓRIA (PMT)

A PMT é uma permeabilização progressiva, não específica da membrana mitocondrial interna, tornando-se gradativamente permeável a prótons, íons, suporte osmótico e pequenas proteínas. Esta permeabilização resulta na perda do potencial de membrana (PM), liberação do Ca^{2+} acumulado e inchamento mitocondrial.

A PMT é dependente da presença de Ca^{2+} no espaço intramitocondrial e pode ser revertida pela adição de quelantes de Ca^{2+} como EGTA, ou redutores ditiólicos logo após a permeabilização (HUNTER e HAWORTH, 1979; ZORATTI e SZABÓ, 1995; CASTILHO et al, 1996). A PMT induzida por Ca^{2+} também pode ser aumentada pela presença concomitante de oxidantes de nucleotídeos de piridina mitocondriais, devido à

depleção do poder redutor mitocondrial e conseqüente estresse oxidativo, provavelmente devido à oxidação de grupamentos tiólicos da membrana mitocondrial interna, promovida por EROs (Figura 4) (LEHNINGER et al., 1978; CASTILHO et al., 1995; KOWALTOWSKI e VERCESI, 1999, KOWALTOWSKI et al., 2001).

Em 1988, CROMPTON et al. descobriram que a PMT podia ser inibida por ciclosporina A (CsA), um imunosupressor, provavelmente devido à ligação da CsA a ciclofilinas da membrana mitocondrial interna, independente de inibição de calcineurinas. Devido a esta regulação pela ciclosporina A, a PMT foi atribuída à abertura de um poro na membrana mitocondrial interna, denominado poro de PMT (CONNERN e HALESTRAP, 1994; NICOLLI et al., 1996; KOWALTOWSKI et al., 2001). Acredita-se que o poro de PMT seja um complexo formado pelo translocador de adenina (ANT), um canal de ânions voltage-dependente (VDAC) e a ciclofilina D. O ANT está localizado na membrana mitocondrial interna, enquanto o VDAC está localizado na membrana mitocondrial externa; estas proteínas atuariam em conjunto para a formação do poro de PMT.

A PMT é um fator desencadeante da morte celular necrótica (GRIFFITHS e HALESTRAP, 1995) apoptótica. A liberação de proteínas pró-apoptóticas para o citoplasma (Figura 4), devido ao inchamento da mitocôndria, com ruptura da membrana externa durante a PMT (GREEN e REED, 1998), evidenciando a importância da disfunção mitocondrial na fisiopatologia celular.

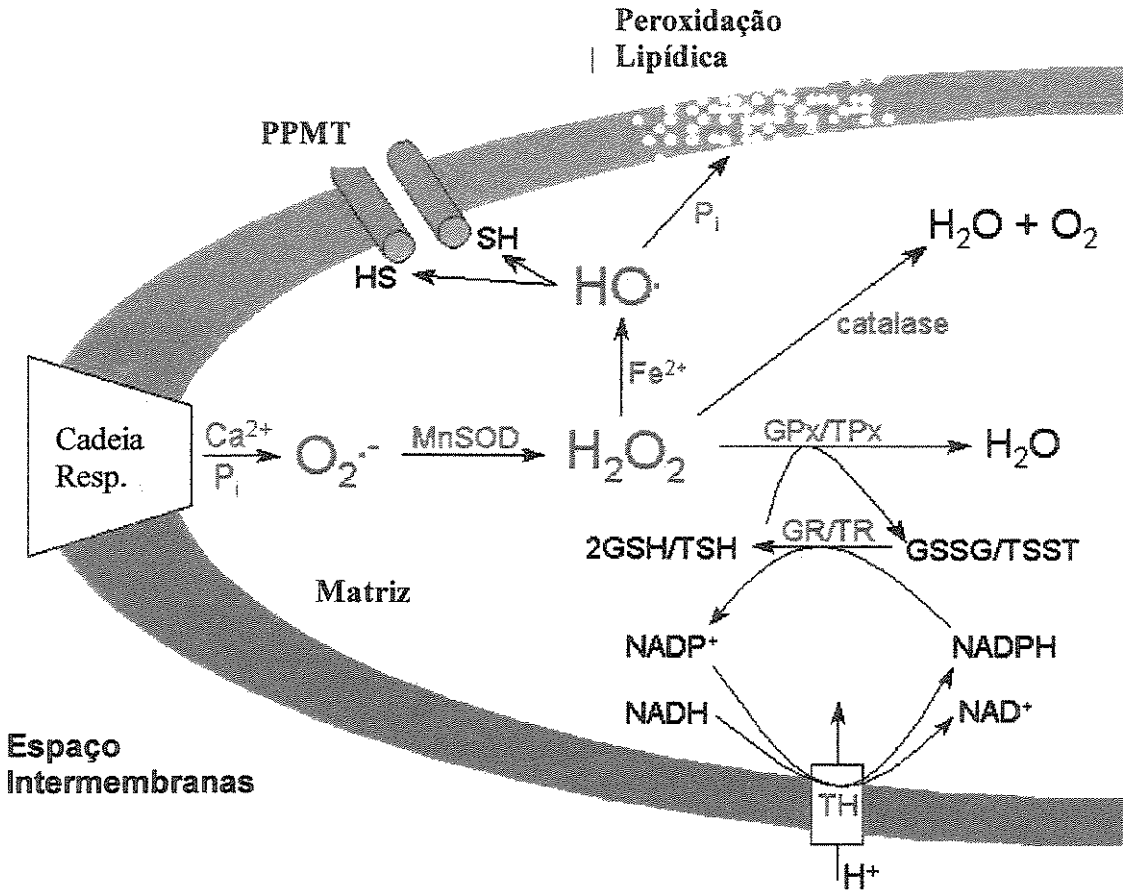


FIGURA 4-Mecanismo proposto para explicar a formação do poro de PMT induzido por Ca^{2+} e estresse oxidativo (adaptado de KOWALTOWSKI et al., 2001). Abreviações: GPx: glutationa peroxidase; GR: glutationa redutase; GSH: glutationa reduzida; GSSG: glutationa oxidada; MnSOD: superóxido dismutase dependente de Mn^{2+}; PPMT: poro de permeabilidade mitocondrial transitória; Resp.: respiratória; TH: NADP transidrogenase; TPx: tioredoxina peroxidase; TSH: tioredoxina reduzida; TSST: tioredoxina oxidada; TR: tioredoxina redutase.

1.7-MECANISMOS DE MORTE CELULAR

Classicamente, a morte celular tem sido dividida em dois tipos, apoptose e necrose. A morte celular por apoptose ocorre de modo regulado, resultando em condensação da cromatina, fragmentação nuclear e formação de "corpos apoptóticos", que

são pequenas vesículas fagocitadas por células jovens (KERR et al., 1972; WYLLIE et al., 1980; MATTSON, 2000; MATTSON et al, 2000). O processo apoptótico é dependente de energia e recentemente mostrou-se que envolve organelas internas, como mitocôndria e retículo endoplasmático. Ao contrário da apoptose, a morte celular necrótica ocorre de modo passivo (não requer ATP). A necrose é caracterizada por perda da homeostase iônica, podendo haver aumento de volume celular e até extravasamento de material citosólico, depleção de energia, perda da síntese protéica e ruptura da membrana plasmática (KERR et al., 1972; WYLLIE et al., 1980; MATTSSON, 2000).

A diferença entre apoptose e necrose foi descoberta por intermédio de células cancerígenas (KERR et al., 1972; WYLLIE et al., 1980) e, posteriormente, esta distinção foi validada para células neuronais (PETTMANN e HENDERSON, 1998). Ambas, apoptose e necrose, ocorrem nas injúrias neuronais e podem ser ativadas num mesmo neurônio (NICOTERA e LIPTON, 1999; LEIST e JÄÄTTELÄ, 2001). Recentemente, outra forma de morte celular foi caracterizada; ativa e regulatória, é chamada de morte celular programada autofágica. O autofagossoma é um vacúolo, originado no retículo endoplasmático, que pode fundir-se com lisossomos, endossomos ou complexo de Golgi e formar um vacúolo chamado autolisossomo (DUNN, 1994; KLIONSKY e EMR, 2000; OHSUMI, 2001). Esta estrutura pode degradar partes do citoplasma e organelas, eventos que geralmente precedem a degradação nuclear na morte autofágica (BURSCH et al., 2000). A morte celular autofágica tem sido observada em desordens neurodegenerativas, como Alzheimer, Parkinson e Huntington, além de isquemia cerebral (CATALDO et al., 1995; ANGLADE et al., 1997; LIPTON, 1999; KEGEL, et al., 2000).

1.7.1-Caspases e proteínas Bcl-2

A apoptose é coordenada por um conjunto de proteases conhecidas como caspases (THORNBERRY e LAZEBNIK, 1998, ZIMMERMANN et al., 2001). Estas proteases são sintetizadas como precursores inativos e quando ativadas clivam substratos intracelulares ativando outras caspases, resultando em uma cascata de caspases que levam à morte celular (THORNBERRY e LAZEBNIK, 1998; WOLF e GREEN, 1999). As caspases envolvidas na apoptose podem ser divididas nas caspases que iniciam a cascata,

caspases-8 e -9, e em caspases que executam a destruição das células, incluindo as caspases-3, -6 e -7 (THORNBERRY e LAZEBNIK, 1998). Várias proteínas podem regular a ativação de caspases, sendo que um número grande destas proteínas tem localização ou interação mitocondrial (RAVAGNAN et al., 2002). A iniciação da cascata de caspases pode ocorrer por duas vias principais. Na primeira, por meio de uma via dependente de fatores mitocondriais (via intrínseca), proteínas da família do Bcl-2 como Bax, Bad, Bak e Bid são mobilizadas ou ativadas e promovem a formação de poros na membrana mitocondrial externa. Por estes poros há a liberação de fatores pró-apoptóticos do espaço intermembranas mitocondrial para o citosol, como o citocromo *c* e o Fator Indutor de Apoptose (AIF). Na segunda via, a morte celular por apoptose é iniciada pela ativação de receptores de morte celular presentes na membrana plasmática (via extrínseca). Estes receptores incluem o receptor de fator de necrose tumoral, o receptor CD95 (Fas, Apo I), dentre outros. A ativação destes receptores irá resultar intracelularmente na ativação da caspase-8, a qual ativará a caspase-3. Pode haver interconexões entre estas duas vias; por exemplo, a caspase-8 pode levar à ativação da proteína Bid, que por sua vez irá promover a formação de poros na membrana mitocondrial externa, juntamente com proteínas Bak ou Bax (GREEN e REED, 1998; LEIST e JÄÄTTELÄ, 2001). No citosol, o citocromo *c* se liga à Apaf-1 ("apoptosis activating factor 1") e à ATP, que se liga à pró-caspase 9, formando o apoptossomo. Este complexo causa uma dimerização e ativação da caspase 9, que por sua vez irá ativar a caspase-3 (LIU et al, 1996; RAVAGNAN et al., 2002). As mitocôndrias também contêm a proteína Smac/DIABLO, que inativa um grupo de proteínas citosólicas responsáveis pela inibição de caspases, provavelmente relacionadas à via intrínseca (DU et al., 2000; RAVAGNAN et al., 2002).

A proteína Bcl-2 foi a primeira proteína a ser identificada (TSUJIMOTO et al., 1985), de uma família de proteínas que regulam a viabilidade celular (VAUX et al., 1988). Algumas destas proteínas promovem a morte celular, como: Bax, Bad, Bcl-X_s e Bid, porém outras proteínas promovem a sobrevivência celular, como Bcl-2 e Bcl-X_L (ADAMS e CORY, 1998; KROEMER et al., 1998).

A proteína Bcl-2 está envolvida em várias vias regulatórias que determinam a sobrevivência celular. Por exemplo, a hiperexpressão de Bcl-2 preserva o potencial de membrana e respiração mitocondrial em mitocôndrias expostas a altas concentrações de

Ca^{2+} (MURPHY et al, 1996). O mecanismo pelo qual a proteína Bcl-2 protege a integridade da membrana mitocondrial interna ainda é controverso (KOWALTOWSKI et al., 2002). Possivelmente, Bcl-2 exerça um efeito direto na estrutura da membrana interna (de JONG et al, 1994) ou indiretamente, tendo um efeito anti-oxidante (BOGDANOV et al., 1999; KOWALTOWSKI et al., 2000) ou modificando o fluxo de moléculas e pequenas proteínas que atravessam as membranas (ANTONSSON et al., 1997; SCHENDEL et al, 1997). A Bcl-2 inibe sobretudo a via apoptótica intrínseca, dependente da liberação de fatores pró-apoptóticos mitocondriais (GREEN e REED, 1998). Em adição, Bcl-2 é um importante inibidor da PMT (MURPHY et al., 1996). A importância da família de proteínas Bcl-2 no sistema nervoso central foi evidenciada a partir de estudos em que neurônios de camundongos transgênicos que hiperexpressam a proteína Bcl-2 possuem proteção parcial contra injúrias decorrentes de isquemia focal (MARTINOU et al., 1994) ou global (KITAGAWA et al., 1998).

2- OBJETIVOS

Anualmente, milhões de pessoas morrem ou passam a sofrer déficit neurológico devido a lesões cerebrais decorrentes de processos isquêmicos. Algumas doenças hereditárias autossômicas recessivas, conhecidas como acidemias orgânicas e caracterizadas por distúrbio no metabolismo de aminoácidos, glicídios ou lipídeos, causam acúmulo tecidual de um ou mais ácidos carboxílicos. Tais acidemias acometem um número menor de indivíduos, que também apresentam lesões no SNC semelhantes às lesões devidas à excitotoxicidade de processos isquêmicos. Ainda não se estabeleceu nenhum tratamento altamente eficaz nestas patologias. Nesta tese, objetivamos compreender a etiologia destas doenças, estudando o metabolismo energético mitocondrial *in vitro* e *in vivo* em modelos experimentais associados a mudanças no metabolismo energético, tais como: modelos de isquemia cerebral e acidemia metilmalônica.

Especificamente, objetivou-se:

- Caracterizar o efeito de Ca^{2+} e Na^{+} na função mitocondrial durante o estresse oxidativo e permeabilidade mitocondrial transitória em modelos *in vitro* (mitocôndrias isoladas, células PC12 e fatias estriatais) e *in vivo* (lesão estriatal por infusão intracerebral de ácido quinolínico). Através destes modelos, pretendemos contribuir para a elucidação do processo de morte celular na isquemia cerebral (estudos *in vitro* e *in vivo*) e acidemias orgânicas (estudos *in vitro*).

3- RESULTADOS

3.1-1°. TRABALHO

Maciel, E. N.; Vercesi, A. E.; G. S.; Castilho, R. F. Oxidative stress in Ca^{2+}-induced membrane permeability transition in brain mitochondria.

Journal of Neurology, 79: 1237-1245, 2001

Journal of Neurochemistry, 2001, **79**, 1237–1245

Oxidative stress in Ca^{2+}-induced membrane permeability transition in brain mitochondria

Evelise N. Maciel, Anibal E. Vercesi and Roger F. Castilho

Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, Brazil

Abstract

Mitochondrial permeability transition (PT) is a non-selective inner membrane permeabilization, typically promoted by the accumulation of excessive quantities of Ca^{2+} ions in the mitochondrial matrix. This phenomenon may contribute to neuronal cell death under some circumstances, such as following brain trauma and hypoglycemia. In this report, we show that Ca^{2+}-induced brain mitochondrial PT was stimulated by Na^+ (10 mM) and totally prevented by the combination of ADP and cyclosporin A. Removal of Ca^{2+} from the mitochondrial suspension by EGTA or inhibition of Ca^{2+} uptake by ruthenium red partially reverted the dissipation of the membrane potential associated with PT. Ca^{2+}-induced brain mitochondrial PT was significantly inhibited by the antioxidant catalase, indicating the participation of reactive oxygen species in this process. An increased detection of reactive oxygen species, measured through dichlorodihydrofluorescein oxidation, was observed after mitochondrial Ca^{2+} uptake. Ca^{2+}-induced dichlorodihydrofluorescein oxidation was enhanced by Na^+ and prevented by ADP and cyclosporin A, indicating that PT enhances mitochondrial oxidative stress. This could be at least in part a consequence of the extensive depletion in NAD(P)H that accompanied this Ca^{2+}-induced mitochondrial PT. NADPH is known to maintain the antioxidant function of the glutathione reductase/peroxidase and thioredoxin reductase/peroxidase systems. In addition, the occurrence of mitochondrial PT was associated with membrane lipid peroxidation. We conclude that PT further increases Ca^{2+}-induced oxidative stress in brain mitochondria leading to secondary damage such as lipid peroxidation.

Keywords: brain mitochondria, calcium, cyclosporin A, excitotoxicity, free radicals, mitochondrial permeability transition.

J. Neurochem. (2001) **79**, 1237–1245.

The maintenance of intracellular Ca^{2+} homeostasis is crucial for neuron survival, and its disruption may be involved in several central nervous system disorders including ischemic and hypoglycemic neuronal death and neurodegeneration in Huntington's disease (for a review see Choi 1995; Fiskum *et al.* 1999). During cytosolic Ca^{2+} overload, the mitochondrion is the main organelle responsible for calcium sequestration. Increased Ca^{2+} concentrations in the mitochondrial matrix may induce a phenomenon called mitochondrial permeability transition (PT), characterized by a cyclosporin A-sensitive non-selective permeabilization of the inner mitochondrial membrane (for a review see Gunter and Pfeiffer 1990; Zoratti and Szabò 1995; Smaili *et al.* 2000; Kowaltowski *et al.* 2001). Mitochondrial PT results in respiration uncoupled from ATP synthesis, organelle swelling, disruption of the outer membrane and release of different apoptogenic factors into the cytosol (Zoratti and Szabò 1995; Green and Reed 1998; Kroemer *et al.* 1998). These factors include cytochrome *c*, apoptosis inducing factor (AIF) and pro-caspases, which promote the execution of apoptosis. A PT-independent mechanism may be also responsible for release of the apoptogenic factor cytochrome *c* after brain mitochondrial Ca^{2+} accumulation (Andreyev and Fiskum 1999; Schild *et al.* 2001). Recent publications have indicated the participation of mitochondrial PT in neuronal death following hypoglycemia (Friberg *et al.* 1998), brain ischemia (Uchino *et al.* 1998; Matsumoto *et al.* 1999) and trauma (Okonkwo and Povlishock 1999;

Received August 1, 2001; revised manuscript received October 1, 2001; accepted October 2, 2001.

Address correspondence and reprint requests to Dr Roger F. Castilho, Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP 13083–970, Brazil. E-mail: roger@fcm.unicamp.br

Abbreviations used: AIF, apoptosis inducing factor; DCF, dichlorofluorescein; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; H_2-DCFDA, dichlorodihydrofluorescein diacetate; L-NAME, *N*ω-nitro-L-arginine methyl ester; PT, permeability transition; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; $\Delta\Psi$, mitochondrial transmembrane electrical potential.

Sullivan *et al.* 2000). However, the participation of mitochondrial PT in excitotoxicity, i.e. glutamate receptor-mediated neuronal cell death, remains controversial (Nieminen *et al.* 1996; Castilho *et al.* 1998; Vergun *et al.* 1999; Brustovetsky and Dubinsky 2000b).

Recent work from our group using isolated rat liver mitochondria has demonstrated an important role for reactive oxygen species (ROS) in Ca^{2+}-induced PT (for a review see Kowaltowski *et al.* 2001). Catalase, ebselen or thioredoxin peroxidase prevent the disruption of liver mitochondrial membrane potential and swelling caused by Ca^{2+} alone or in the presence of inducers such as *t*-butyl hydroperoxide, inorganic phosphate and fatty acids (Valle *et al.* 1993; Castilho *et al.* 1995; Kowaltowski *et al.* 1996, 1998; Catisti and Vercesi 1999). In addition, no PT occurs in liver mitochondria in the absence of molecular O_2. It is proposed that Ca^{2+} ions are involved in the mechanism of mitochondrial PT pore opening by: (i) binding to the anionic head of membrane cardiolipins, stimulating the production of superoxide anion radicals and, hence, H_2O_2, by the respiratory chain (Valle *et al.* 1993; Castilho *et al.* 1995; Grijalba *et al.* 1999); (ii) stimulating the Fenton reaction through matrix iron mobilization (Castilho *et al.* 1995) and (iii) binding to membrane proteins that regulate PT pore opening (Bernardi *et al.* 1992; Zoratti and Szabò 1995).

In this paper, we study the involvement of oxidative stress in Ca^{2+}-induced membrane PT in brain mitochondria. Our results indicate that ROS are implicated in Ca^{2+}-induced brain mitochondrial PT. In addition, PT results in mitochondrial oxidative stress, represented by increased detection of ROS and secondary mitochondrial oxidative damage.

Materials and methods

Isolation of rat brain mitochondria

Forebrain mitochondria were isolated as described by Brustovetsky and Dubinsky (2000a,b), with minor modifications. Wistar rats were fasted overnight prior to killing by decapitation. The brains were rapidly removed (within 1 min) and put into ice-cold isolation buffer I containing 225 mannitol, 75 mM sucrose, 1 mM K^+-EGTA, 0.1% bovine serum albumin (BSA; free fatty acid) and 10 mM K^+-HEPES pH 7.2. The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was minced using surgical scissors and then extensively washed. The tissue was then homogenized in a power-driven, tight fitting Potter-Elvehjem homogenizer with Teflon pestle. The resulting suspension was centrifuged for 7 min at 2000 *g* in a Beckman JA 20 rotor. After centrifugation the supernatant was recentrifuged for 10 min at 12 000 *g*. The pellet was resuspended in 'isolation buffer II' containing 225 mannitol, 75 mM sucrose, 1 mM K^+-EGTA, and 10 mM K^+-HEPES pH 7.2 and recentrifuged at 12 000 *g* for 10 min. The supernatant was decanted and the final pellet gently washed and resuspended in 'isolation buffer II' devoid of EGTA, at an approximate protein concentration of 30–40 mg/mL. The respiratory control ratio (state 3/state 4 respiratory rates) was over 3.0, measured using succinate and glutamate as substrates.

Preparation of dichlorodihydrofluorescein (H_2-DCF)-loaded forebrain mitochondria

Rat forebrain mitochondria were obtained as described above, except that the pellet obtained after the second centrifugation was resuspended to a protein concentration of 10 mg/mL in 'isolation buffer II' containing 10 μM dichlorodihydrofluorescein diacetate (H_2-DCFDA). The mitochondrial suspension was incubated at 30°C for 15 min and then recentrifuged at 12 000 *g* for 10 min. The supernatant was decanted and the pellet gently washed and diluted in isolation buffer II devoid of EGTA, at an approximate protein concentration of 20–30 mg/mL.

Standard incubation procedure

The experiments were carried out at 28°C, with continuous magnetic stirring, in a standard reaction medium containing 100 mM sucrose, 65 mM KCl, 10 mM K^+-HEPES buffer (pH 7.2), 20 μM EGTA, 1 mM P_i, 5 mM glutamate and 5 mM succinate. Other additions are indicated in the figure legends. We used 20 μM EGTA to buffer contaminating free Ca^{2+} (8–10 μM) present in the reaction medium. Under our conditions, when 80 μM Ca^{2+} was added to the experiments, the free Ca^{2+} concentration in the medium was about 70 μM, as calculated according to Fabiato and Fabiato (1979). The results shown are representative of a series of at least four experiments, using different mitochondrial preparations. The results were reproduced within 15% of variation.

Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

Mitochondrial $\Delta\Psi$ was estimated through fluorescence changes of safranin O (5 μM) that were recorded on a model F-4010 Hitachi spectrofluorometer (Hitachi Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm (Åkerman and Wikström 1976).

Determination of Ca^{2+} movements

Variations in the concentration of free extramitochondrial Ca^{2+} were followed by measuring changes in the absorbance spectrum of arsenazo III (40 μM), using an SLM Aminco DW2000 spectrophotometer (SLM Instruments Inc., Urbana, IL, USA) set at the wavelength pair 665–685 nm (Scarpa 1979). The addition of mitochondria to reaction medium containing arsenazo III resulted in a rapid increase in absorbance (Figs 1C, 2B and 4C). This increase was observed even in the presence of a high EGTA concentration (500 μM; results not shown), indicating that was a phenomenon probably related to changes in medium turbidity and not due to the presence of contaminant Ca^{2+} in the mitochondrial suspension.

Organelles in intact cells show a very slow accumulation of safranine (20–30 min; Åkerman and Jarvisalo 1980; Vercesi *et al.* 1991), and those within synaptosomes do not take up Ca^{2+} promptly under our experimental conditions. Thus, there would be no significant interference with the safranine and arsenazo measurements unless the synaptosomes were permeabilized.

Estimation of reactive oxygen species production

Mitochondrial generation of reactive oxygen species (H_2O_2) was determined spectrofluorometrically, using the membrane-permeable fluorescent dye H_2-DCFDA (1 μM; LeBel *et al.* 1992; Garcia-Ruiz *et al.* 1997). Fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm. Calibration was performed by adding known concentrations of dichlorofluorescein (DCF), the product of H_2-DCF oxidation.

Determination of NAD(P) redox state

The oxidation or reduction of pyridine nucleotides in the mitochondrial suspension was followed using a Hitachi F-4010 spectrofluorometer operating at excitation and emission wavelengths of 366 and 450 nm, respectively, with slit widths of 5 nm. Diamide (1 mM) was added at the end of each experiment to fully oxidize the pyridine nucleotides which remained in the reduced state.

Determination of thiobarbituric acid-reactive reactive substances (TBARS)

TBARS production by mitochondria was measured according to Buege and Aust (1978). Briefly, 0.4 mL samples were taken after 15 min incubation at 30°C in standard reaction medium and mixed with 0.4 mL of 1% thiobarbituric acid in 50 mM NaOH, 0.2 mL de 20% of H_3PO_4 and 40 μL of 10 N NaOH. The mixture was heated at 90°C for 20 min in the presence of 1 mM butylated hydroxy-toluene. After cooling, 1.5 mL of butanol was added to the solution. The mixture was shaken and centrifuged at 2000 r.p.m. during 3 min. The optical density of the organic layer was determined at 535 nm. Under these conditions, the molar extinction coefficient used to calculate TBARS concentrations is 1.56×10^5 $M^{-1}cm^{-1}$ (Buege and Aust 1978).

Chemicals

Most chemicals, including ADP, arsenazo III, catalase (10 000 units/mg), cyclosporin A, diamide, dithiothreitol, ebselen, EGTA, FCCP, glutamic acid, HEPES, safranine O, succinic acid, ruthenium red and thiobarbituric acid, were obtained from Sigma Chemical Company (St Louis, MO, USA). H_2-DCFDA was purchased from Molecular Probes (Eugene, OR, USA). All other reagents were commercial products of the highest purity grade available.

Results

In the present work, PT in isolated brain mitochondria was assessed by measuring the transmembrane electrical potential ($\Delta\Psi$) and mitochondrial Ca^{2+} release (Brustovetsky and Dubinsky 2000a,b). The results in Fig. 1(A) show that the classical PT inhibitors (Zoratti and Szabò 1995) cyclosporin

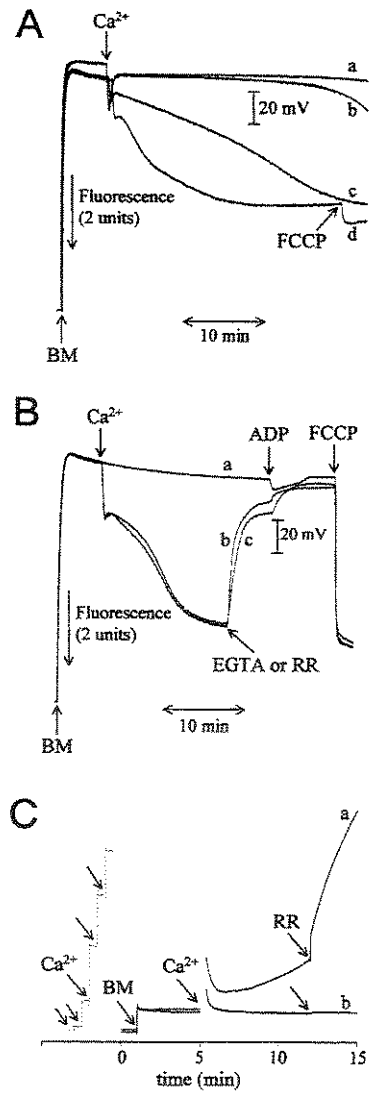


Fig. 1 Ca^{2+}-induced mitochondrial transmembrane electrical potential ($\Delta\Psi$) dissipation and Ca^{2+} release: effect of mitochondrial PT inhibitors. Isolated rat forebrain mitochondria (BM) (0.5 mg/mL) were incubated at 28°C in standard reaction medium containing 5 μM safranin O to estimate $\Delta\Psi$ (panels A and B) or 40 μM arsenazo III to measure extramitochondrial free Ca^{2+} concentrations (panel C).In panel A, BM were added to reaction medium containing 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (line a), 200 μM ADP and 1 μg/mL oligomycin (line b), 1 μM cyclosporin A (line c) or no other additions (line d). Ca^{2+} (80 μM, lines a–d) and 1 μM FCCP (line d) were added where indicated by the arrows. In panel B, BM were added to reaction medium and 80 μM Ca^{2+} (lines b and c), 1 mM EGTA (line b), 1 μM ruthenium red (RR; line c), 200 μM ADP (lines a–c) and 1 μM FCCP were added where indicated by the arrows. In panel C, BM were added to reaction medium containing 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (line b) or no other additions (line a). Ca^{2+} (80 μM, lines a and b) and RR (1 μM, lines a and b) were added where indicated by the arrows. The dotted line represents standard additions of Ca^{2+} (10 μM) to the reaction medium in the absence of BM.

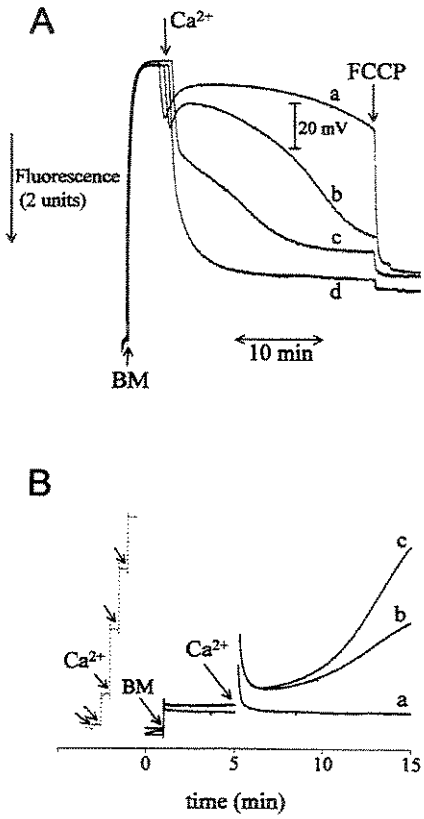


Fig. 2 Inhibitory effect of exogenous catalase on Ca^{2+}-induced mitochondrial $\Delta\Psi$ dissipation and Ca^{2+} release. BM (0.5 mg/mL) were incubated at 28°C in standard reaction medium containing 5 μM safranin O to estimate $\Delta\Psi$ (panel A) or 40 μM arsenazo III to measure extramitochondrial free Ca^{2+} concentrations (panel B). In panel A, BM were added to reaction medium containing 2 μM catalase (lines a and c) or no other additions (lines b and d). Ca^{2+} (70 μM, lines a and b or 90 μM, lines c and d) and 1 μM FCCP were added where indicated by the arrows. In panel B, BM were added to reaction medium containing 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (line a), 2 μM catalase (line *b*) or no other additions (line c). Ca^{2+} (80 μM, lines a-c) was added where indicated by the arrow. The dotted line represents standard additions of Ca^{2+} (10 μM) to the reaction medium in the absence of BM.

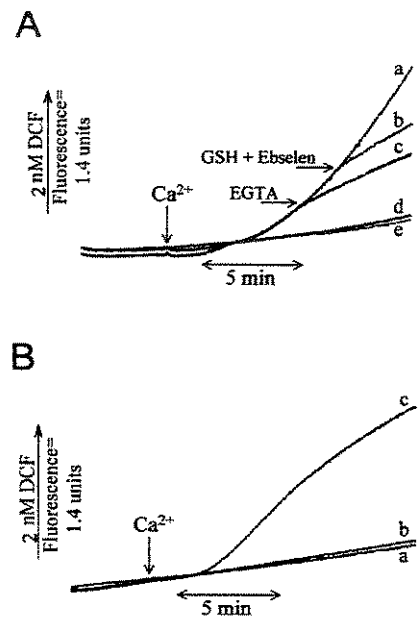


Fig. 3 Ca^{2+}-induced increased detection of mitochondrial generation of reactive oxygen species. In panel A, BM (0.5 mg/mL) were added to standard reaction medium at 28°C containing 1 μM H_2-DCFDA in the presence of 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (line d) or no other additions (lines a-c). Ca^{2+} (80 μM), EGTA (1 mM), GSH (200 μM) and ebselen (10 μM) were added where indicated by the arrows (lines a-d). Line *e* represents a control experiment without addition of Ca^{2+}. In panel B, H_2-DCF-loaded BM (see Materials and methods) were added to reaction medium at 28°C in the presence of 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (line b) or no other additions (line a and c). Ca^{2+} (80 μM) was added where indicated by the arrow (lines b and c). Line a represents a control experiment without the addition of Ca^{2+}.

A (line c) or ADP plus oligomycin (line b) only partially inhibit $\Delta\Psi$ dissipation caused by Ca^{2+} (line d). A complete inhibition of Ca^{2+}-induced $\Delta\Psi$ dissipation was only obtained when ADP, oligomycin and cyclosporin A were present simultaneously (line a). Under this last condition, only a transient decrease of $\Delta\Psi$, due to electrophoretic mitochondrial Ca^{2+} uptake, was observed. These results are in accordance with previous publications showing only partial inhibition of brain mitochondrial PT by cyclosporin A (Kristal and Dubinsky 1997; Brustovetsky and Dubinsky 2000a,b; Kristian *et al.* 2000). Figure 1(B) shows that removal of Ca^{2+} from the brain mitochondrial suspension by EGTA (line b) or inhibition of mitochondrial Ca^{2+} uptake by ruthenium red (line c) partially reverts the Ca^{2+}-induced $\Delta\Psi$ dissipation. $\Delta\Psi$ reversibility by EGTA or ruthenium red was not complete and a further increase in $\Delta\Psi$ was obtained by the addition of ADP. In contrast, the addition of ADP to a control experiment (line a) caused a transient decrease in $\Delta\Psi$ related to ADP phosphorylation. In Fig. 1(C), measuring the release of intramitochondrial Ca^{2+} determined mitochondrial membrane permeabilization. Ca^{2+} addition is followed by a fast mitochondrial Ca^{2+} uptake, and subsequent release of mitochondrial Ca^{2+} to the reaction medium (line a). Mitochondrial Ca^{2+} release was inhibited by cyclosporin A, ADP and oligomycin (line b), indicating the participation of PT in this process. Since mitochondrial Ca^{2+} release occurs simultaneously with

Ca^{2+} uptake, blocking mitochondrial Ca^{2+} uptake with ruthenium red resulted in a faster increase of extramitochondrial Ca^{2+} in the absence of PT inhibitors (line a).

In Fig. 2, the effect of the antioxidant catalase was tested on brain mitochondrial PT. The presence of catalase significantly inhibited $\Delta\Psi$ dissipation (Fig. 2A, line a) when a lower Ca^{2+} concentration was added (70 μM; line b). However a smaller inhibition of $\Delta\Psi$ dissipation was obtained by catalase (line c) when a higher Ca^{2+} concentration was added (90 μM; line d). The presence of the nitric oxide inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 3–5 mM) resulted in no inhibition of Ca^{2+}-induced $\Delta\Psi$ dissipation (result not shown). The results depicted in Fig. 2(B) show that catalase significantly inhibited (line b) Ca^{2+}-induced mitochondrial Ca^{2+} release in the absence of PT inhibitors (line c).

In order to further investigate the participation of oxidative stress in Ca^{2+}-induced brain mitochondrial PT, we measured mitochondrial reactive oxygen species (ROS) production (Fig. 3). H_2-DCFDA is oxidized mainly by H_2O_2 and peroxynitrite (Reynolds and Hastings 1995; LeBel *et al.* 1992; Jakubowski and Bartosz 2000) generating DCF, which is highly fluorescent. A fast increase in the rate of DCF production was observed 3–4 min after the addition of Ca^{2+} (Fig. 3A, line a), indicating a higher detection of ROS under this condition. This increase in mitochondrial ROS production was completely inhibited by the PT inhibitors cyclosporin A, ADP and oligomycin (line d). It is important to emphasize that under our experimental conditions, no significant mitochondrial swelling, measured by changes in absorbance at 520 nm, was observed in the presence of Ca^{2+} (results not shown). This excludes the possibility that the increase in DCF fluorescence could be an experimental artefact related to a decrease in light scattering of the mitochondrial suspension. The presence of synaptosomes in our mitochondrial suspension may mask absorbance changes related to Ca^{2+}-induced PT (Brustovetsky and Dubinsky 2000a,b). Ca^{2+} removal by EGTA (line c) and the antioxidants ebselen plus glutathione (line b) blocked Ca^{2+}-induced increase in mitochondrial ROS detection. Using H_2-DCF-loaded mitochondria (Fig. 3B), we obtained similar results to those observed when H_2-DCFDA was initially present only in the extramitochondrial medium (Fig. 3A). These results suggest that Ca^{2+}-induced increase in mitochondrial ROS production is not related to PT-promoted access of H_2-DCFDA to intramitochondrial ROS production sites.

As, under some pathological conditions, increase in cytoplasmic free Ca^{2+} in neurons is accompanied by an

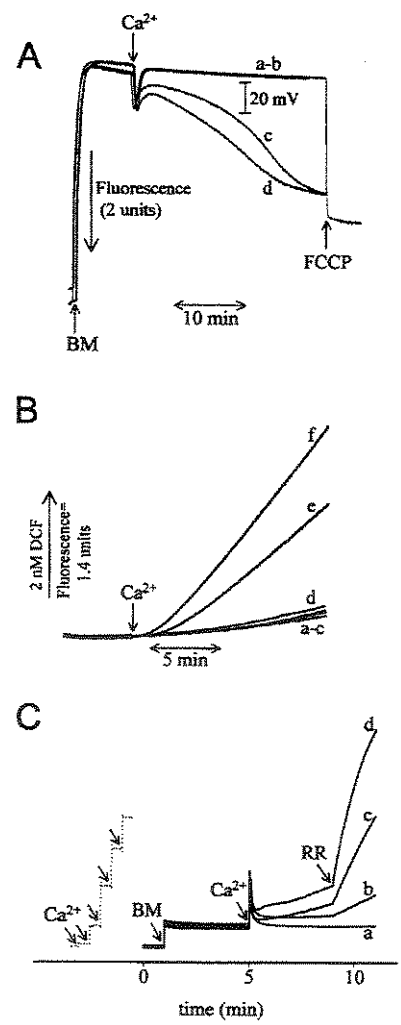


Fig. 4 Effect of Na^+ on Ca^{2+}-induced $\Delta\Psi$ dissipation (panel A), mitochondrial ROS generation (panel B) and mitochondrial Ca^{2+} release (panel C). In panel A, BM (0.5 mg/mL) were added to reaction medium at 28°C containing 5 μM safranine O in the presence of 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (lines a and b) and 10 mM NaCl (lines b and d). Ca^{2+} (80 μM) and 1 μM FCCP were added where indicated by the arrows (lines a-d). In panel B, BM (0.5 mg/mL) were added to reaction medium containing 1 μM H_2-DCFDA at 28°C in the presence of 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (lines c and d) and 10 mM NaCl (line b, d and f). Ca^{2+} (80 μM) was added where indicated by the arrows (lines c-f). Lines a and b represent control experiments without the addition of Ca^{2+}. In panel C, BM (0.5 mg/mL) were added to reaction medium containing 40 μM arsenazo III in the presence of 200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A (lines a and b) and 10 mM NaCl (lines b and d). Ca^{2+} (80 μM) and 1 μM RR were added where indicated by the arrows to the experiments represented by lines a-d. The dotted line represents standard additions of Ca^{2+} (10 μM) to the reaction medium in the absence of BM.

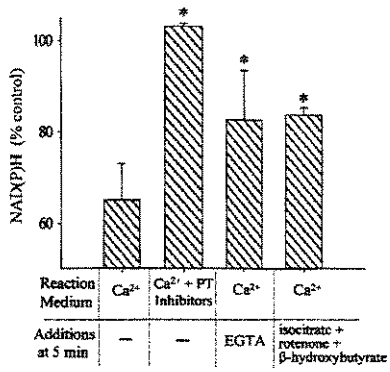


Fig. 5 Ca^{2+}-induced brain mitochondrial PT and NAD(P)H oxidation. BM (0.5 mg/mL) were added to reaction medium at 28°C in the presence of 80 μM Ca^{2+} and PT inhibitors (200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A), as indicated in the figure. At 5 min, 1 mM EGTA, 100 μM isocitrate, 4 μM rotenone and/or 5 mM β-hydroxybutyrate were added to the experiments as indicated. At 18 min, the remaining reduced pyridine nucleotides were estimated by the addition of the oxidant diamide (1 mM). The data are presented as percent of control experiments in which BM were incubated in the presence of 1 mM EGTA and diamide was added after 18 min. Values represent averages of 4 experiments (± SD), using different mitochondrial preparations. * $p < 0.01$, *post hoc* Bonferroni's test compared with the experiment in which only Ca^{2+} was added.

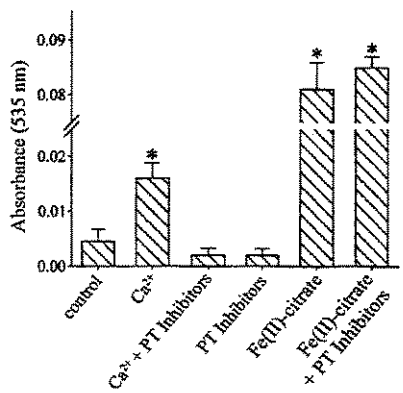


Fig. 6 Ca^{2+}-induced brain mitochondrial PT and TBARS formation. BM (1.0 mg/mL) were incubated at 30°C in standard reaction medium for 15 min in the presence of 160 μM Ca^{2+} and/or PT inhibitors (200 μM ADP, 1 μg/mL oligomycin and 1 μM cyclosporin A) as indicated in the figure. In the experiments of this figure, as in others (Figs 1 and 3,4,5), 160 nmol Ca^{2+}/mg protein was used. A larger mitochondrial protein concentration was used in this experiment (1 mg/mL) to increase the sensitivity of TBARS detection. Fe^{2+} (50 μM) plus 2 mM citrate were used to induce lipid peroxidation independent of mitochondrial PT. Values represent averages of 4 experiments (± SD), using different mitochondrial preparations. * $p < 0.01$, *post hoc* Bonferroni's test compared with control.

increase in Na^+ concentrations (e.g. excitotoxicity; Choi 1987), the effect of Na^+ on Ca^{2+}-induced brain mitochondrial PT and ROS generation was studied (Fig. 4). We found that Na^+ stimulates Ca^{2+}-induced $\Delta\Psi$ dissipation (panel A, line d compared with line c), mitochondrial ROS detection (panel B, line f compared with line e) and mitochondrial Ca^{2+} release sensitive to PT inhibitors (panel C, line d compared with line c). As expected, the presence of Na^+ increased the release of intramitochondrial Ca^{2+}, even in the presence of PT inhibitors (line b), due to the activation of the Na^+/Ca^{2+} exchanger in brain mitochondria (Crompton *et al.* 1978; Nicholls and Scott 1980). However, the rate of mitochondrial Ca^{2+} release observed in line d was faster than the rate of mitochondrial Ca^{2+} release observed in line c (PT-mediated Ca^{2+} release in the absence of Na^+) plus line b (Na^+/Ca^{2+} exchanger-mediated Ca^{2+} release). These results suggest that Na^+ increases PT-mediated mitochondrial Ca^{2+} release.

In Fig. 5, we studied the effect of Ca^{2+} and PT on endogenous reduced pyridine nucleotides (NAD(P)H) in brain mitochondria. Incubation of brain mitochondria in the presence of Ca^{2+}, during 15 min, resulted in approximately 40% loss of reduced pyridine nucleotides. Interestingly, the PT inhibitors cyclosporin A, ADP and oligomycin completely prevented the Ca^{2+}-induced loss of reduced pyridine nucleotide. The addition of the Ca^{2+} chelator EGTA or the NAD(P) reductants isocitrate and β-hydroxybutyrate at 5 min incubation partially prevents the effect of Ca^{2+} on reduced pyridine nucleotides.

Mitochondrial lipid oxidation was quantified in order to study a possible correlation between PT-induced increased detection of ROS and membrane damage (Fig. 6). Incubation of brain mitochondria in the presence of Ca^{2+} increases the basal content of TBARS 3–4 fold, in a process completely prevented by the PT inhibitors cyclosporin A, ADP and oligomycin. PT inhibitors did not inhibit mitochondrial lipid oxidation induced by Fe(II)-citrate (Castilho *et al.* 1994), indicating that these compounds do not have direct antioxidant properties.

Discussion

The PT in rat liver and heart mitochondria can be mediated by a concerted action between Ca^{2+} and ROS leading to oxidation of membrane protein thiols (Valle *et al.* 1993; Castilho *et al.* 1995; Grijalba *et al.* 1999; Kowaltowski *et al.* 2001). We have previously shown that Ca^{2+} stimulates mitochondrial ROS production (Valle *et al.* 1993; Castilho *et al.* 1995; Grijalba *et al.* 1999), which promotes the oxidation and cross-linkage of mitochondrial membrane protein thiol groups, leading to PT in liver and heart mitochondria (Fagian *et al.* 1990; Valle *et al.* 1993; Castilho *et al.* 1995; Kowaltowski *et al.* 1996). In the present work, the participation of oxidative stress in Ca^{2+}-induced brain

mitochondrial PT was evidenced by: (i) a significant inhibition of both Ca^{2+}-induced $\Delta\Psi$ dissipation and mitochondrial Ca^{2+} release in the presence of catalase (Fig. 2), (ii) an increased detection of ROS following PT (Fig. 3) and (iii) depletion of endogenous reduced pyridine nucleotides and oxidation of membrane lipids associated with PT (Figs 5 and 6).

The measurements of H_2-DCFDA oxidation detected an oxidative stress situation that was totally inhibited by PT pore inhibitors (Fig. 3). It is possible that Ca^{2+} stimulates the generation of ROS at specific sites of the inner mitochondrial membrane, where they attack protein thiols opening PT pore (Valle *et al.* 1993; Castilho *et al.* 1995; Kowaltowski *et al.* 1996; Kowaltowski *et al.* 1998). These ROS may not be detected before mitochondrial PT by H_2-DCFDA oxidation measurements due to their local and instantaneous effect on membrane proteins (Stadtman 1990).

Our results showed an increase in mitochondrial generation of ROS following membrane depolarization, thus indicating a condition of oxidative stress. This contrasts with the expected decrease in ROS production after membrane depolarization under control conditions (Boveris and Chance 1973; Skulachev 1996). Mitochondrial membrane depolarization increases the rate of electron transfer in the respiratory chain, thereby decreasing the steady state reduction of electron carriers and the oxygen tension. This minimizes superoxide formation at the level of complexes I and III (Boveris and Chance 1973; Skulachev 1996).

The PT-induced oxidative stress observed in the present work may be the result of either a stimulation of mitochondrial ROS production or a failure of mitochondrial antioxidant systems. Mitochondrial PT may result in structural alterations of the inner mitochondrial membrane that affect respiratory chain function, including coenzyme Q mobility (Nohl *et al.* 1996; Grijalba *et al.* 1999), and favor monoelectronic oxygen reduction (superoxide radical generation) at intermediate steps of the respiratory chain. In addition, PT-induced cytochrome *c* release from the mitochondrial intermembrane space results in stimulation of mitochondrial generation of superoxide (Cai and Jones 1998). PT is also expected to impair the mitochondrial antioxidant systems glutathione reductase/peroxidase and thioredoxin reductase/peroxidase that depend on NADPH to reduce H_2O_2 to water. Mitochondrial PT results in dissipation of the transmembrane proton electrochemical gradient and, at low membrane potentials, the NADP transhydrogenase cannot sustain high levels of mitochondrial reducing power (NADPH, reduced glutathione and thioredoxin), favoring oxidative stress (Vercesi 1987; Hoek and Rydstrom 1988). Moreover, the opening of mitochondrial PT pores results in loss of endogenous NAD(P)H and glutathione to the extramitochondrial medium (Igbavboa *et al.* 1989). Indeed, our results showed a decrease, partially due to oxidation, in the concentration of reduced pyridine nucleotides following brain mitochondrial PT (Fig. 5). Higher generation of ROS following mitochondrial membrane depolarization in neurons exposed to excitotoxic conditions has also been reported (Tenneti *et al.* 1998; Luetjens *et al.* 2000). A similar phenomenon was recently described in intact cardiac myocytes exposed to photoactivated tetramethylrhodamine derivatives (Zorov *et al.* 2000). PT-induced brain mitochondrial oxidative stress, with consequent peroxidation of membrane lipids (Fig. 6), may result in the impairment of mitochondrial oxidative phosphorylation and in irreversible inner membrane permeabilization.

Interestingly, Ca^{2+}-induced brain mitochondrial PT was stimulated by Na^+ (10 mM; Fig. 4). This suggests that, under conditions in which an increase in cytoplasmic free Ca^{2+} is accompanied by an increase in Na^+ concentration, such as that found in excitotoxicity (Choi 1987), there is a stimulation of Ca^{2+}-induced brain mitochondrial PT and oxidative stress. This result is in accordance with a previous report (Dykens 1994) showing that Na^+ increases ROS generation by cerebral and cerebellar isolated mitochondria in the presence of Ca^{2+}. On the other hand, Kristal *et al.* (2000) did not observe a significant stimulatory effect of Na^+ on brain mitochondrial PT, estimated by mitochondrial swelling. This observation is in apparent contrast with our results showing that Na^+ potentiates Ca^{2+}-induced brain mitochondrial $\Delta\Psi$ dissipation and Ca^{2+} release. Probably, the swelling measurements secondary to the entry of the osmotic support can not detect small alterations in inner mitochondrial membrane permeability (Gunter and Pfeiffer 1990), explaining why Kristal *et al.* (2000) did not observe a stimulatory effect of Na^+ on Ca^{2+}-induced brain mitochondrial PT.

Finally, we suggest that PT-induced brain mitochondrial oxidative stress and dysfunction may participate, together with the release of mitochondrial apoptogenic signal molecules into the cytosol, in the cascade of events (Tenneti *et al.* 1998; Andreyev and Fiskum 1999; Castilho *et al.* 1999; Luetjens *et al.* 2000; Petersen *et al.* 2000) that determine neuronal cell death under conditions associated with cytosolic Ca^{2+} overload.

Acknowledgements

We thank Dr Alicia J. Kowaltowski for critical reading of the manuscript and Mrs Elisangela J. Silva for the preparation of rat forebrain mitochondria. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundo de Apoio ao Ensino e Pesquisa (FAEP-UNICAMP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). ENM is supported by a FAPESP fellowship.

References

Åkerman K. E. and Jarvisalo J. O. (1980) Effects of ionophores and metabolic inhibitors on the mitochondrial membrane potential within isolated hepatocytes as measured with the safranine method. *Biochem. J.* **192**, 183–190.

Åkerman K. E. and Wikström M. K. (1976) Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett.* **68**, 191–197.

Andreyev A. and Fiskum G. (1999) Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain versus liver. *Cell Death Differ.* **6**, 825–832.

Bernardi P., Vassanelli S., Veronese P., Colonna R., Szabo I. and Zoratti M. (1992) Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. *J. Biol. Chem.* **267**, 2934–2939.

Boveris A. and Chance B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134**, 707–716.

Brustovetsky N. and Dubinsky J. M. (2000a) Dual responses of CNS mitochondria to elevated calcium. *J. Neurosci.* **20**, 103–113.

Brustovetsky N. and Dubinsky J. M. (2000b) Limitations of cyclosporin A inhibition of the permeability transition in CNS mitochondria. *J. Neurosci.* **20**, 8229–8237.

Buege J. A. and Aust. S. D. (1978) Microsomal lipid peroxidation. *Meth. Enzymol.* **52**, 302–310.

Cai J. and Jones D. P. (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome *c* loss. *J. Biol. Chem.* **273**, 11401–11404.

Castilho R. F., Meinicke A. R., Almeida A. M., Hermes-Lima M. and Vercesi A. E. (1994) Oxidative damage of mitochondria induced by Fe (II) citrate is potentiated by Ca^{2+} and includes lipid peroxidation and alterations in membrane proteins. *Arch. Biochem. Biophys.* **308**, 158–163.

Castilho R. F., Kowaltowski A. J., Meinicke A. R., Bechara E. J. H. and Vercesi A. E. (1995) Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic. Biol. Med.* **18**, 479–486.

Castilho R. F., Hansson O., Ward M. W., Budd S. L. and Nicholls D. G. (1998) Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurosci.* **18**, 10277–10286.

Castilho R. F., Ward M. W. and Nicholls D. G. (1999) Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurochem.* **72**, 1394–1401.

Catisti R. and Vercesi A. E. (1999) The participation of pyridine nucleotides redox state and reactive oxygen in the fatty acid-induced permeability transition in rat liver mitochondria. *FEBS Lett.* **464**, 97–101.

Choi D. W. (1987) Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* **7**, 369–379.

Choi D. W. (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci.* **18**, 58–60.

Crompton M., Moser R., Ludi H. and Carafoli E. (1978) The interrelations between the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur. J. Biochem.* **82**, 25–31.

Dykens J. A. (1994) Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: implications for neurodegeneration. *J. Neurochem.* **63**, 584–591.

Fabiato A. and Fabiato F. (1979) Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)* **75**, 463–505.

Fagian M. M., Pereira-da-Silva L., Martins I. S. and Vercesi A. E. (1990) Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca^{2+} plus prooxidants. *J. Biol. Chem.* **265**, 19955–19960.

Fiskum G., Murphy A. N. and Beal M. F. (1999) Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. *J. Cereb. Blood Flow Metab.* **19**, 351–369.

Friberg H., Ferrand-Drake M., Bengtsson F., Halestrap A. P. and Wieloch T. (1998) Cyclosporin A, but not FK 506, protects mitochondrial and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J. Neurosci.* **18**, 5151–5159.

Garcia-Ruiz C., Colell A., Mari M., Morales A. and Fernandez-Checa J. C. (1997) Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J. Biol. Chem.* **272**, 11369–11377.

Green D. R. and Reed J. C. (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312.

Grijalba M. T., Vercesi A. E. and Schreier S. (1999) Ca^{2+}-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca^{2+}-stimulated generation of reactive oxygen species by the respiratory chain. *Biochemistry* **38**, 13279–13287.

Gunter T. E. and Pfeiffer D. R. (1990) Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258**, C755–C786.

Hoek J. B. and Rydstrom J. (1988) Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem. J.* **254**, 1–10.

Igbavboa U., Zwizinski C. W. and Pfeiffer D. R. (1989) Release of mitochondrial matrix proteins through a Ca^{2+}-requiring cyclosporin-sensitive pathway. *Biochem. Biophys. Res. Commun.* **161**, 619–625.

Jakubowski W. and Bartosz G. (2000) 2,7-dichlorofluorescin oxidation and reactive oxygen species: what does it measure? *Cell Biol. Int.* **24**, 757–760.

Kowaltowski A. J., Castilho R. F., Grijalba M. T., Bechara E. J. and Vercesi A. E. (1996) Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation. *J. Biol. Chem.* **271**, 2929–2934.

Kowaltowski A. J., Netto L. E. S. and Vercesi A. E. (1998) The thiol-specific antioxidant enzyme prevents mitochondrial permeability transition. Evidence for the participation of reactive oxygen species in this mechanism. *J. Biol. Chem.* **273**, 12766–12769.

Kowaltowski A. J., Castilho R. F. and Vercesi A. E. (2001) Mitochondrial permeability transition and oxidative stress. *FEBS Lett.* **495**, 12–15.

Kristal B. S. and Dubinsky J. M. (1997) Mitochondrial permeability transition in the central nervous system: induction by calcium cycling-dependent and-independent pathways. *J. Neurochem.* **69**, 524–538.

Kristal B. S., Staats P. N. and Shestopalov A. I. (2000) Biochemical characterization of the mitochondrial permeability transition in isolated forebrain mitochondria. *Dev. Neurosci.* **22**, 376–383.

Kristian T., Gertsch J., Bates T. E. and Siesjo B. K. (2000) Characteristics of the calcium-triggered mitochondrial permeability transition in nonsynaptic brain mitochondria: effect of cyclosporin A and ubiquinone O. *J. Neurochem.* **74**, 1999–2009.

Kroemer G., Dallaporta B. and Resche-Rigon M. (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol.* **60**, 619–642.

LeBel C. P., Ischiropoulos H. and Bondy S. C. (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5**, 227–231.

Luetjens C. M., Bui N. T., Sengpiel B., Munstermann G., Poppe M.,

Krohn A. J., Bauerbach E., Krieglstein J. and Prehn J. H. (2000) Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. *J. Neurosci.* **20**, 5715–5723.

Matsumoto S., Friberg H., Ferrand-Drake M. and Wieloch T. (1999) Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.* **19**, 736–741.

Nicholls D. G. and Scott I. D. (1980) The regulation of brain mitochondrial calcium-ion transport. The role of ATP in the discrimination between kinetic and membrane-potential-dependent calcium-ion efflux mechanisms. *Biochem. J.* **186**, 833–889.

Nieminen A. L., Petrie T. G., Lemasters J. J. and Selman W. R. (1996) Cyclosporin A delays mitochondrial depolarization induced by *N*-methyl-D-aspartate in cortical neurons: evidence of the mitochondrial permeability transition. *Neuroscience* **75**, 993–997.

Nohl H., Gille L., Schonheit K. and Liu Y. (1996) Conditions allowing redox-cycling ubisemiquinone in mitochondria to establish a direct redox couple with molecular oxygen. *Free Radic. Biol. Med.* **20**, 207–213.

Okonkwo D. O. and Povlishock J. T. (1999) An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury. *J. Cereb. Blood Flow Metab.* **19**, 443–451.

Petersen A., Castilho R. F., Hansson O., Wieloch T. and Brundin P. (2000) Oxidative stress, mitochondrial permeability transition and activation of caspases in calcium ionophore A23187-induced death of cultured striatal neurons. *Brain Res.* **857**, 20–29.

Reynolds I. J. and Hastings T. G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J. Neurosci.* **1995**, 3318–3327.

Scarpa A. (1979) Measurements of cation transport with metallochromic indicators. *Meth. Enzymol.* **56**, 301–338.

Schild L., Keilhoff G., Augustin W., Reiser G. and Striggow F. (2001) Distinct Ca^{2+} thresholds determine cytochrome c release or permeability transition pore opening in brain mitochondria. *FASEB J.* **15**, 565–567.

Skulachev V. P. (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q. Rev. Biophys.* **29**, 169–202.

Smaili S. S., Hsu Y.-T., Youle R. J. and Russel J. T. (2000) Mitochondria in Ca^{2+} signaling and apoptosis. *J. Bioenerg. Biomembr.* **32**, 35–46.

Stadtman E. R. (1990) Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic. Biol. Med.* **9**, 315–325.

Sullivan P. G., Thompson M. and Scheff S. W. (2000) Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury. *Exp. Neurol.* **161**, 631–637.

Tenneti L., D'Emilia D. M., Troy C. M. and Lipton S. A. (1998) Role of caspases in *N*-methyl-D-aspartate-induced apoptosis in cerebrocortical neurons. *J. Neurochem.* **71**, 946–959.

Uchino H., Elmer E., Uchino K., Li P. A., He Q. P., Smith M. L. and Siesjo B. K. (1998) Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. *Brain Res.* **812**, 216–226.

Valle V. G. R., Fagian M. M., Parentoni L. S., Meinicke A. R. and Vercesi A. E. (1993) The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants. *Arch. Biochem. Biophys.* **307**, 1–7.

Vercesi A. E. (1987) The participation of NADP, the transmembrane potential and the energy-linked NAD (P) transhydrogenase in the process of Ca^{2+} efflux from rat liver mitochondria. *Arch. Biochem. Biophys.* **252**, 171–178.

Vercesi A. E., Bernardes C. F., Hoffmann M. E., Gadelha F. R. and Docampo R. (1991) Digitonin permeabilization does not affect mitochondrial function and allows the determination of the mitochondrial membrane potential of. *Trypanosoma Cruzi Situ. J. Biol. Chem.* **266**, 14431–14434.

Vergun O., Keelan J., Khodorov B. I. and Duchen M. R. (1999) Glutamate-induced mitochondrial depolarisation and perturbation of calcium homeostasis in cultured rat hippocampal neurones. *J. Physiol.* **519**, 451–466.

Zoratti M. and Szabò I. (1995) The mitochondrial permeability transition. *Biochim. Biophys. Acta* **1241**, 139–176.

Zorov D. B., Filburn C. R., Klotz L. O., Zweier J. L. and Sollott S. J. (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J. Exp. Med.* **192**, 1001–1014.

 Journal of Neurochemistry, **79**, 1237–1245

3.2-2º. TRABALHO

Maciel, E. N.; Kaminski Schierle, G. S.; Hansson, O.; Brundin, P.; Castilho, R. F. Cyclosporin A and Bcl-2 do not inhibit quinolinic acid-induced striatal excitotoxicity in rodents.

Experimental Neurology, 183: 430-437, 2003.

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Experimental Neurology 183 (2003) 430–437

Experimental Neurology

www.elsevier.com/locate/yexnr

Cyclosporin A and Bcl-2 do not inhibit quinolinic acid-induced striatal excitotoxicity in rodents

Evelise N. Maciel,[a] Gabriele S. Kaminski Schierle,[b,1] Oskar Hansson,[b] Patrik Brundin,[b] and Roger F. Castilho[a,*]

[a] *Departamento de Patologia Clinica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP 13083-970, Brazil*
[b] *Section for Neuronal Survival, Wallenberg Neuroscience Center, BMC A10, Lund University, 221 84 Lund, Sweden*

Received 18 July 2002; revised 16 January 2003; accepted 25 February 2003

Abstract

Mitochondrial permeability transition (MPT) is a nonselective inner membrane permeabilization that contributes to neuronal cell death under circumstances such as brain trauma, ischemia, and hypoglycemia. Here we study the participation of MPT and the Bcl-2-sensitive apoptotic cell death pathway in glutamate receptor-mediated excitotoxicity. Intrastriatal infusions of the *N*-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid caused massive striatal neurodegeneration in both rats and mice. Interestingly, transgenic mice overexpressing human Bcl-2 and rats systemically treated with cyclosporin A did not exhibit reduced sensitivity to quinolinic acid-induced striatal toxicity. Both Bcl-2 and cyclosporin A are inhibitors of MPT; in addition Bcl-2 also inhibits apoptotic stimuli-mediated release of mitochondrial apoptogenic factors. Isolated brain mitochondria from cyclosporin A-treated rats showed resistance to Ca^{2+}-induced dissipation of the membrane potential, indicating protection against MPT. We conclude that quinolinic acid-mediated striatal excitotoxicity is not dependent on MPT and Bcl-2-sensitive apoptotic cell death pathways.

Keywords: Bcl-2; Calcium; Cyclosporin A; Mitochondrial permeability transition; NMDA; Quinolinic acid; striatum

Introduction

Excitotoxicity is a central nervous system process in which an increased glutamate release in response to, e.g., hypoglycemia, ischemia, or trauma results in neuronal necrosis or apoptosis (for reviews, see Rothman and Olney, 1986; Albin and Greenamyre, 1992; Choi, 1996; Nicholls and Budd, 2000). This glutamate-mediated cell death is largely, but not entirely, mediated by the Ca^{2+} and Na^{+} permeant *N*-methyl-D-aspartate (NMDA)-selective glutamate receptor. While Na^{+} influx in neurons is mainly associated with cellular swelling, Ca^{2+} influx is correlated with cellular toxicity (Choi, 1987). During cytosolic Ca^{2+} overload, the mitochondrion is the main organelle responsible for Ca^{2+} sequestration (Nicholls and Budd, 2000). Increased Ca^{2+} concentration in the mitochondrial matrix may induce a phenomenon called mitochondrial permeability transition (MPT), characterized by a cyclosporin A-sensitive nonselective permeabilization of the inner mitochondrial membrane (for reviews, see Zoratti and Szabò, 1995; Kowaltowski et al., 2001). MPT is also inhibited by high transmembrane membrane potential ($\Delta\psi_m$), adenine nucleotides, Mg^{2+}, acidic pH, antioxidants, and Bcl-2, an antiapoptotic protein mainly found in the outer mitochondrial membrane under normal conditions (Zoratti and Szabò, 1995; Kowaltowski et al., 2001). The inner mitochondrial membrane permeabilization caused by MPT results in loss of matrix components, impairment of oxidative phosphorylation, and swelling of the organelle, with consequent outer membrane rupture and release of different apoptogenic factors into the cytosol (Zoratti and Szabò, 1995; Crompton, 1999; Kowaltowski et al., 2001). Mitochondrial apoptogenic factors include cytochrome *c* (Liu et al., 1996).

* Corresponding author. Fax: +55-19-3788-9434.
E-mail address: roger@fcm.unicamp.br (R.F. Castilho).
[1] Present address: Cambridge Center for Brain Repair, University of Cambridge, Forvie Site, Robinson Way, CB2 2PY Cambridge, UK.

0014-4886/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0014-4886(03)00165-1

apoptosis-inducing factor (Susin et al., 1996), and a protein known both as Diablo and Smac, that neutralizes a set of caspase inhibitors known as inhibitor of apoptosis proteins (IAPs) (Du et al., 2000). Furthermore, cytosolic calcium overload activates different enzymes involved in cell death (phospholipase A_2, calcineurin, proteases, and endonucleases) (Leist and Nicotera, 1998; Orrenius et al., 1996; Coyle and Puttfarcken, 1993; Wang et al., 1999) and induces cellular oxidative stress (Coyle and Puttfarcken, 1993; Lafon-Cazal et al., 1993; Reynolds and Hasting, 1995; Castilho et al., 1999).

The inhibitory property of cyclosporin A on MPT is related to its binding to inner mitochondrial membrane cyclophilin D in a process associated with inhibition of the peptidylprolyl *cis-trans* isomerase activity (Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991; Nicolli et al., 1996). Although the immunosuppressive activity of cyclosporin A is mediated by inhibition of the phosphatase calcineurin, this mechanism is not involved in MPT inhibition (Griffiths and Halestrap, 1991). Studies focused on experimental models of neurodegeneration report that cyclosporin A inhibits cell death in cultured neurons (Nieminen et al., 1996; Schinder et al., 1996; Petersen et al., 2000) and in rat brain (Uchino et al., 1998; Friberg et al., 1998), implicating the participation of MPT. The mechanism of MPT inhibition by Bcl-2 is less well understood. Experiments using cells that overexpress Bcl-2 suggest that the inhibition of MPT promoted by Bcl-2 is mediated by enhancing H^+ efflux in the presence of membrane potential-loss-inducing stimuli (Shimizu et al., 1998), by maintaining mitochondrial pyridine nucleotides in a reduced redox state (Kowaltowski et al., 2000) and/or by preventing oxidation of protein thiol groups (Zamzami et al., 1998). Bcl-2 could also inhibit the release of mitochondrial apoptogenic factors in a mechanism independent of MPT inhibition (for a review, see Martinou and Green, 2001).

In this paper, we study the role of MPT and the Bcl-2-sensitive apoptotic cell death pathway in striatal lesions in rodents induced by quinolinic acid, a potent NMDA receptor agonist. We attempted to inhibit MPT in vivo either by systemic treatment of rats with cyclosporin A or by using mice overexpressing Bcl-2.

Materials and methods

Disruption of the blood-brain barrier

Female Wistar rats weighing 250 g were fasted overnight with access to water before surgery. Six days before intrastriatal infusion of quinolinic acid/vehicle or forebrain mitochondrial isolation the rats were anesthetized with pentobarbital sodium (40 mg/kg, ip). In order to disrupt the blood-brain-barrier a 10-μl Hamilton syringe (cannula outer diameter 450 μm) was inserted in the right dorsal hippocampus and saline (2 μl; 0.9 % NaCl) was stereotactically injected over 2 min at the following coordinates: 3.5 mm caudal to bregma; 1.3 mm lateral to midline; 3.0 mm ventral from the dural surface; with the tooth-bar set at zero (Uchino et al., 1998).

Striatal lesions in rats, cyclosporin A treatment, and tissue preparation

Six days following rupture of the blood-brain barrier, the rats were anesthetized with pentobarbital sodium (50 mg/kg, ip). Intrastriatal injections of 50 nmol (2 μl) of quinolinic acid from a 10-μl Hamilton syringe were performed in the right hemisphere at the following stereotaxic coordinates: 1.3 mm rostral to bregma; 2.7 mm lateral to midline; 4.5 mm ventral from the dural surface; with the tooth-bar set at zero. The toxin was injected over 2 min, and afterward the cannula was left in place for an additional 2 min.

The rats received daily intraperitoneal injections of vehicle ($n = 7$) or 15 mg kg^{-1} cyclosporin A ($n = 7$) starting 4 days before the quinolinic acid injection and continuing for 4 days after injection of the excitotoxin. On the day of surgery, the rats received two injections of vehicle or cyclosporin A, at 1 h before and at 4 h after the quinolinic acid infusion. During pilot experiments we observed that a higher cyclosporin A dose, 20 mg Kg^{-1} day^{-1} for 8 days, was toxic and resulted in loss of body weight or death of the rats. Cyclosporin A (50 mg/ml; Sandimmune, Novartis, Switzerland) was diluted to 10 mg/ml in 0.9% NaCl. Cremophor oil was diluted five times in 0.9% NaCl and was used as vehicle.

At 4 days after the quinolinate injection the animals were deeply anesthetized with pentobarbital sodium (80 mg/kg, ip) and transcardially perfused with saline for 2 min followed by 250 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and kept in the same formaldehyde solution for 24 h prior to transferring them into 20% sucrose/0.1 M phosphate buffer for 36 h. The brains were cut on a freezing microtome into 3 series of 40-μm-thick coronal sections through the striatum before processing them for Fluoro-Jade staining.

Striatal lesions in mice and tissue processing

We used transgenic mice in which neurons overexpress the human Bcl-2 protein under the control of the neuron-specific enolase (NSE) promoter, as described by Martinou et al. (1994). Males (C57BL/6) heterozygous for the transgene were crossed with C57BL/6 females. The presence of the *bcl-2* transgene was confirmed by polymerase chain reaction (Dubois-Dauphin et al., 1994). Wild-type littermate ($n = 7$) and transgenic female mice with Bcl-2 overexpression ($n = 7$) weighing 30 g were fasted overnight with access to water before surgery. Under equithesin anesthesia (3 ml/kg, ip), the mice received intrastriatal injections of 50 nmol (1 μl) of quinolinic acid from a 2-μl Hamilton syringe at the following stereotaxic coordinates: 0.7 mm rostral to

bregma; 1.8 mm lateral to midline; 2.4 mm ventral from the dural surface; with the tooth-bar set at zero. The toxin was injected for 2 min, and the cannula was left in place for an additional 2 min.

Four weeks after the quinolinate injection, the animals were deeply anesthetized with pentobarbital sodium (80 mg/kg, ip) and transcardially perfused with 30 ml saline, followed by 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and maintained in the same fixative for 4 h before being transferred into 20% sucrose/0.1 M phosphate buffer for 24 h. The brains were sectioned coronally through the striatum using a freezing microtome into 3 series of 40-μm-thick sections before being processed for DARPP-32 immunohistochemistry.

Fluoro-Jade

Fluoro-Jade staining was performed as described by Schmued et al. (1997). Briefly, fixed brain sections were mounted, dried, and immersed in 100% ethanol, followed by 70% ethanol. Sections were then treated with 0.06% potassium permanganate for 15 min. After rinsing, sections were immersed in Fluoro-Jade (0.001% Fluoro-Jade/0.1% acetic acid; Histo-Chem Inc., Jefferson, AR) for 30 min.

DARPP-32 immunohistochemistry

Free-floating sections were processed for DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of a molecular weight of 32 kDa) immunohistochemistry. Free-floating sections were incubated in 10% horse serum/0.2% Triton X-100/0.1 M PBS for 1 h at room temperature, followed by a reaction with an antibody against DARPP-32 (1:20,000; kindly donated by Drs. P. Greengard and H. Hemmings, Weil Medical College, New York, NY) for 48 h at 4°C. Sections were incubated with a biotinylated secondary antibody (1:200) for 1 h, and bound antibody was visualized using the ABC system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), with 3,3′-diaminobenzidine as chromogen.

Cell counting and data analysis

The number of DARPP-32-positive cells in the striatum was assessed on blind-coded slides with a semiautomated stereological system [Olympus C.A.S.T. Grid system (version 1.10), composed of an Olympus BX50 microscope and a *X-Y-Z* step motor stage run by a computer]. This sampling technique is not affected by tissue volume changes, does not require reference volume determination (West et al., 1991), and is considered to provide the most accurate estimates of cell numbers (Coggesshall and Lekan, 1996). The area of the striatum was delineated and a counting frame was randomly placed within the striatum to mark the first area to be sampled. The frame was systematically moved through the striatum. The number of positive cells was then extrapolated according to a stereological algorithm (West et al., 1991). The areas containing Fluoro-Jade-positive cells were delineated and the lesion volume was determined using the same equipment as described above (West et al., 1991). All data were analyzed by unpaired two-tailed Student's *t* test and presented as means ± SD.

Isolation of rat forebrain mitochondria from control and cyclosporin A-treated rats

The blood-brain barrier was disrupted in all rats as described above, except for the rats used for the experiments shown in Fig. 3B. The rats received daily intraperitoneal injections of vehicle or cyclosporin A (15 mg/kg) starting 5 days before forebrain mitochondrial isolation, with the last injection made 10 h before the mitochondrial isolation. Forebrain mitochondria were isolated from control and cyclosporin A-treated rats as described by Rosenthal et al. (1987) with minor modifications. Rats were killed by decapitation, and their brains rapidly removed (within 1 min) and put into 10 ml of ice-cold "isolation buffer" containing 225 mannitol, 75 mM sucrose, 1 mM K^+-EGTA, 0.1 % bovine serum albumin (BSA; free fatty acid), 10 mM K^+-Hepes, pH 7.2, and 5 mg protease. The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000*g* in a Beckman JA 20 rotor. After centrifugation the supernatant was recentrifuged for 8 min at 12,000*g*. The pellet was resuspended in 20 ml of isolation buffer containing 80 μl of 10% digitonin and recentrifuged for 8 min at 12,000*g*. The supernatant was decanted and the final pellet gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 30–40 mg/ml. The respiratory control ratio (state 3/state 4 respiratory rates) was over 4.0, measured using succinate and glutamate as substrates.

Measurements of mitochondrial transmembrane electrical potential ($\Delta\psi$)

Mitochondrial $\Delta\psi$ was estimated through fluorescence changes of safranin O (5 μM) recorded on a Model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm (Åkerman and Wikström, 1976).

Chemicals

Most chemicals, including ADP, digitonin, EGTA, FCCP, glutamic acid, Hepes, protease type VIII, quinolinic

acid, safranine O, and succinic acid were obtained from Sigma Chemical Company (St. Louis, MO), Fluoro-Jade was purchased from Histo-Chem Inc.

Results

Transgenic mice overexpressing human Bcl-2 and rats systemically treated with cyclosporin A are not protected against quinolinic acid-induced striatal lesion

It has been shown that transgenic mice overexpressing human Bcl-2 under the control of the neuron-specific enolase promoter are partially resistant to brain lesion induced by ischemia, trauma, inhibitors of mitochondrial respiratory chain, and neuronal death secondary to axotomy (Dubois-Dauphin et al., 1994; Martinou et al., 1994; Kitagawa et al., 1998; Raghupathi et al., 1998; Yang et al., 1998; Bogdanov et al., 1999). To study if overexpression of Bcl-2 could render striatal neurons resistant to excitotocicity in vivo we tested the sensitivity of these mice to quinolinic acid, a potent agonist of NMDA receptor (Foster et al., 1988). The quinolinic acid-induced striatal lesions in mice were evaluated as loss of DARPP-32-immunopositive neurons (striatal projection neurons). Four weeks after quinolinic acid infusion, only around 50% of the DARPP-32-immunostained neurons remained in the striatum in wild-type control mice compared with the contralateral nonlesioned side (Fig. 1). Transgenic mice overexpressing human Bcl-2 exhibited lesions of a similar size, indicating that overexpression of Bcl-2 in vivo does not render striatal neurons resistant to excitotoxicity.

In situations when the blood-brain barrier is compromised, systemic treatment of rats with cyclosporin A results in resistance to brain lesions induced by ischemia, hypoglycemia, 3-nitropropionic acid, and trauma (Friberg et al., 1998; Uchino et al., 1998; Matsumoto et al., 1999; Okonkwo and Povlishock, 1999; Leventhal et al., 2000). In our experimental paradigm, rats were initially subjected to brain surgery to disrupt the blood-brain barrier prior to receiving daily injections of cyclosporin A for 8 days, starting 4 days before intrastriatal quinolinic acid infusion. The treatment with cyclosporin A did not cause a reduction in the number of Fluoro-Jade-positive dead or dying striatal neurons (Fig. 2), suggesting that inhibition of MPT does not protect neurons against excitotoxic death in vivo. Moreover, no toxic effect of cyclosporin A was observed on striatal neuron morphology. This was evidenced by the lack of alterations in both striatal volume and neuronal number in the striatum contralateral to the lesion (results not shown).

MPT in isolated rat brain mitochondria: Protective effects of systemic treatment with cyclosporin A and of in vitro micromolar concentrations of ADP and ATP

In order to study if systemic treatment with cyclosporin A inhibits MPT in brain mitochondria, we assessed transmembrane electrical potential after exposure to high $[Ca^{2+}]$ in isolated brain mitochondria from vehicle and cyclosporin A-treated rats (Fig. 3A). Isolated mitochondria from cyclosporin A-treated rats were strongly resistant to Ca^{2+}-induced decrease of transmembrane electrical potential (Fig. 3A).

The lack of participation of MPT in quinolinic acid-induced striatal lesion could be explained by the presence of endogenous inhibitors of this phenomenon in vivo. A potent inhibition of Ca^{2+}-induced Ca^{2+} release from brain mitochondrial and inner membrane permeabilization by adenine nucleotides has been previously reported (Nicholls and Scott, 1980; Rottenberg and Marbach, 1990; Brustovetsky and Dubinsky, 2000). Indeed the results shown in Fig. 3B indicate that micromolar concentrations of ATP (line d) and ADP (line c) inhibit Ca^{2+}-induced decrease of transmembrane electrical potential. No protective effect was observed in the presence of AMP (line b). Since brain mitochondrial preparation has considerable adenylate kinase and ATPase activities, part of the ATP added to the experiment can be converted to ADP, making it difficult to establish the K_i for these adenine nucleotides in Ca^{2+}-induced brain MPT (Rottenberg and Marbach, 1990).

Discussion

MPT has been implicated in cell death in several experimental models for neuronal disorders, including brain hypoglycemia, ischemia, and trauma (Friberg et al., 1998; Matsumoto et al., 1999; Uchino et al., 1998; Okonkwo and Povlishock, 1999). Since excitotoxicity is believed to contribute to neuronal cell death under these conditions (for a review, see Fiskum et al., 1999), we investigated whether MPT is involved in excitotoxic brain damage in rats and mice following infusion of quinolinic acid, a potent endogenous agonist of NMDA receptor. We studied rats systemically treated with the MPT inhibitor cyclosporin A (Uchino et al., 1998) and mice overexpressing the endogenous MPT inhibitor Bcl-2 (Martinou et al., 1994; Murphy et al., 1996). Our results did not reveal any protective effect of Bcl-2 (Fig. 1) or cyclosporin A (Fig. 2) on quinolinic acid-induced striatal toxicity, indicating that excitotoxic neuronal cell death is not dependent on MPT in this model.

The present results are consistent with our earlier findings showing that the MPT inhibitors bongkrekic acid and cyclosporin A do not inhibit glutamate-induced cytoplasmic Ca^{2+} deregulation in cultured cerebellar granule cells (Castilho et al., 1998). A very limited protective effect of cyclosporin A on glutamate-induced death of hippocampal cultured neurons was also recently reported by Brustovetsky and Dubinsky (2000). The lack of effect of cyclosporin A in quinolinic acid-induced striatal lesion in rats was probably not due to a failure of MPT inhibition in vivo, since isolated brain mitochondria from cyclosporin A-treated rats showed

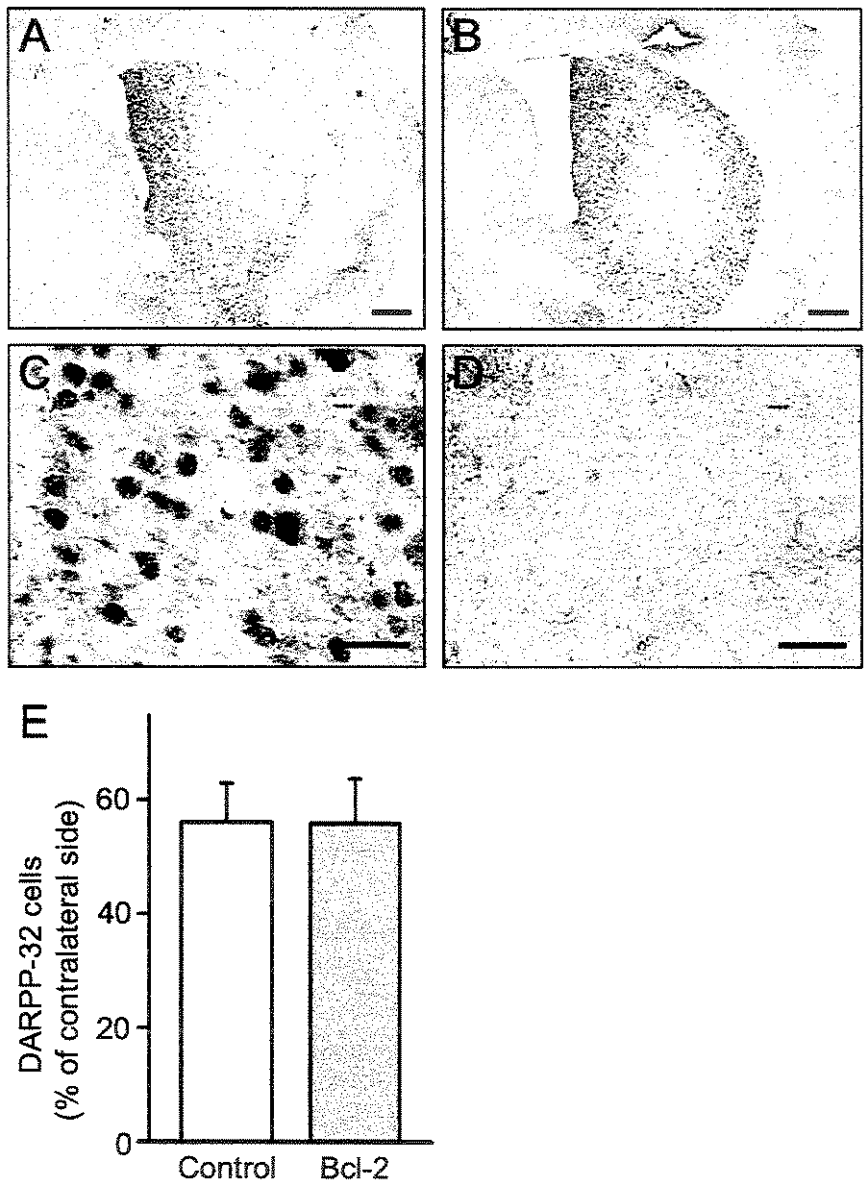


Fig. 1. Transgenic mice overexpressing human Bcl-2 are not protected against quinolinic acid-induced striatal toxicity. Photomicrographs of striatal sections (A–D) were prepared from brains 4 weeks after intrastriatal quinolinic acid injection, processed for DARPP-32 immunohistochemistry. Sections from wild-type (A) and transgenic mice (B) show massive loss of striatal DARPP-32 neurons. (C and D) Photomicrographs of contralateral (unlesioned side) and ipsilateral (closest to the site of quinolinic acid injection) striatal sections of a wild-type mouse, respectively. (E) Quantification of the number of striatal DARPP-32-positive neurons. The number of positive neurons was counted in the five coronal sections closest to the site of injection and is expressed as a mean (error bar = SD) percentage of the number of positive cells on the contralateral unlesioned side (n = 7 transgenic mice; n = 7 wild-type mice). [Bar = 0.5 mm (A and B) and 30 μm (C and D)].

resistance to Ca^{2+}-induced MPT when compared with isolated organelles from vehicle-treated rats (Fig. 3A).

Bcl-2 overexpression in cultured cell lines results in protection against Ca^{2+}-induced MPT (Murphy et al., 1996; Shimizu et al., 1998; Zamzami et al., 1998; Kowaltowski et al., 2000) and apoptotic stimuli involving release of mitochondrial apoptogenic factors such as cytochrome *c* (Martinou and Green, 2001). Several reports show that transgenic mice overexpressing Bcl-2 display marked protection against neuronal cell death promoted by oxidative stress (Merad-Saidoune et al., 1999), mitochondrial respiratory chain inhibition (Yang et al., 1998; Bogdanov et al., 1999), serum deprivation (Schierle et al., 1999), and staurosporine (Schierle et al., 1999). On the other hand, Yang et al (2000) did not observe inhibition of MPT in isolated liver mitochondria from transgenic mice overexpressing human Bcl-2 under the control of liver-specific enolase. Our results with transgenic mice overexpressing Bcl-2 suggest that quino-

linic acid-induced striatal cell death is not dependent on Bcl-2-sensitive apoptotic cell death pathways.

In the experiments with both mice and rats, we chose to inject a concentration and volume of quinolinic acid that lesions approximately 50% of the striatal volume. We expected that this size of lesion was suitable if we wanted to observe a protective effect of cyclosporin A or Bcl-2 on striatal neurons. Although the lesion (cell death) is complete around the needle track, i.e., around the quinolinic acid infusion site, there is a clear penumbra where a few neurons survive and where one could expect to first observe the effect of neuroprotective agents, should it be mild (Nakao et al., 1996; Dirnagl et al., 1999). Thus, if CsA or Bcl-2 were to display any neuroprotective effect at low quinolinic acid concentrations, they would be expected to inhibit cell death in the penumbra.

The results indicating that MPT is not involved in quinolinic acid-induced cell death were unexpected, since NMDA hyperstimulation results in a high Ca^{2+} and Na^{+} influx followed by mitochondrial Ca^{2+} accumulation (Nicholls and Budd, 2000) and increased cellular production of reactive oxygen species (Castilho et al., 1999), situations that strongly favor MPT (Zoratti and Szabò, 1995; Kowaltowski et al., 2001; Maciel et al., 2001). One possible explanation for the lack of participation of MPT in our experimental model is the presence of endogenous inhibitors of this phenomenon in vivo, such as adenine nucleotides. In fact, we observed a potent inhibition of Ca^{2+}-induced MPT in vitro by micromolar concentrations of ADP or ATP (Fig. 3B). Another possibility is that under our experimental conditions the mitochondrial pathways of neuronal cell death, including MPT, are bypassed by alternative intracellular mechanisms that eventually result in cell death. Finally, we propose that situations where ATP and ADP are severely depleted, such as brain ischemia (Uchino et al., 1998; Matsumoto et al., 1999) and hypoglycemia (Friberg et al., 1998), would favor the participation of MPT in excitotoxicity.

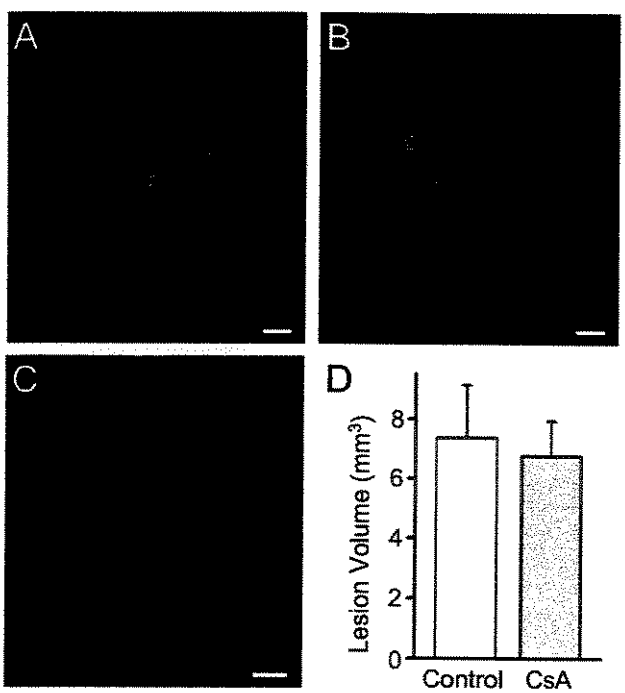


Fig. 2. Rats systemically treated with cyclosporin A (CsA) are not protected against quinolinic acid-induced striatal toxicity. Photomicrographs of striatal sections (A–C) were prepared from brains 4 days after intrastriatal quinolinic acid injection, labeled with the fluorescent cell death marker Fluoro-Jade. Sections from vehicle-treated (A) and cyclosporin A-treated (B) rats contain numerous stained cells. (C) Photomicrograph (closest to the site of quinolinic acid injection) of a striatal section of a control rat. (D) Quantification of lesion volume. The lesion volume was determined and expressed as mean ± SD (n = 7 vehicle-injected rats; n = 7 cyclosporin A-treated rats). [Bar = 0.5 mm (A and B) and 60 μm (C and D)].

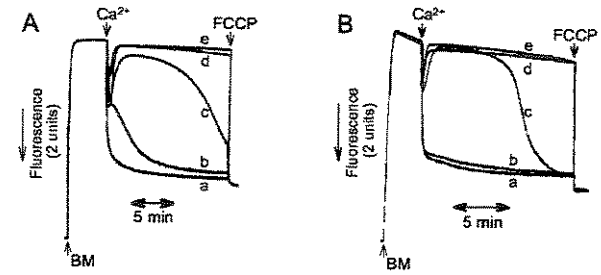


Fig. 3. Protective effects of systemic treatment with cyclosporin A (A) and in vitro micromolar concentrations of ADP and ATP (B) on Ca^{2+}-induced mitochondrial transmembrane electrical potential ($\Delta\psi$) dissipation. Isolated brain mitochondria (BM) (0.5 mg/ml) were incubated at 28°C in standard reaction medium (100 mM sucrose, 65 mM KCl, 10 mM K^{+}-Hepes buffer (pH 7.2), 20 μM EGTA, 1 mM P_i, 5 mM glutamate, and 5 mM succinate) containing 5 μM safranine O to estimate $\Delta\psi$. Mg^{2+} (2.5 mM) was also present in the reaction medium, except for the experiments shown in panel A, to minimize the formation of complexes between Ca^{2+} and adenine nucleotides. (A) BM from vehicle- (lines a, b, and e) and cyclosporin A-treated (lines c and d) rats were added to reaction medium. Ca^{2+} (80 μM, lines b and d, or 100 μM, (lines a, c, and e) and 1 μM FCCP (lines a–e) were added where indicated by arrows. Line e represents an experiment where BM were added to reaction medium containing 200 μM ADP, 1 μg/ml oligomycin, and 1 μM cyclosporin A. (B) BM were added to reaction medium containing 100 μM AMP (line b), 100 μM ADP (line c), 100 μM ATP (line d), 200 μM ADP, 1 μg/ml oligomycin plus 1 μM cyclosporin (line e), or no other additions (line a). Ca^{2+} (120 μM) and 1 μM FCCP were added where indicated by the arrows. The results shown are representative of a series of at least four experiments, using different mitochondrial preparations. The results were reproduced within 10% of variation.

Acknowledgments

The authors are grateful to Professors Anibal Vercesi and Gilberto Fernandes (State University of Campinas, Brazil) for valuable discussions and for providing laboratory facilities for the realization of many experiments of this study. We acknowledge Mrs. Elisângela J. Silva for the preparation of rat forebrain mitochondria. We are grateful to Prof. J.C. Martinou for providing Bcl-2 overexpressing breeder

mice. This study was supported by grants from the Swedish Medical Research Council (to P.B.), the Swedish Society for Medical Research (G.S.K.S.), the Parkinsonsförbundets Forskningsfond (P.B. and G.S.K.S.), Crafoord Stiftelse (G.S.K.S.), FAPESP (R.F.C.), and CNPq (R.F.C.). E.N.M. is supported by a FAPESP fellowship.

References

Åkerman, K.E., Wikström, M.K., 1976. Safranine as a probe of the mitochondrial membrane potential. FEBS Lett. 68, 191–197.

Albin, R.L., Greenamyre, J.T., 1992. Alternative excitotoxic hypotheses. Neurology 42, 733–738.

Bogdanov, M.B., Ferrante, R.J., Mueller, G., Ramos, L.E., Martinou, J.C., Beal, M.F., 1999. Oxidative stress is attenuated in mice overexpressing Bcl-2. Neurosci. Lett. 262, 33–36.

Brustovetsky, N., Dubinsky, J.M., 2000. Limitations of cyclosporin A inhibition of the permeability transtion in CNS mitochondria. J. Neurosci. 20, 8229–8237.

Castilho, R.F., Hansson, O., Ward, M.W., Budd, S.L., Nicholls, D.G., 1998. Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurosci. 18, 10277–10286.

Castilho, R.F., Ward, M.W., Nicholls, D.G., 1999. Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurochem. 72, 1394–1401.

Choi, D.W., 1987. Ionic dependence of glutamate neurotoxicity. J. Neurosci. 7, 369–379.

Choi, D.W., 1996. Ischemia-induced neuronal apoptosis. Curr. Opin. Neurobiol. 6, 667–672.

Coggeshall, R.E., Lekan, H.A., 1996. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. J. Comp. Neurol. 364, 6–15.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689–695.

Crompton, M.R., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233–249.

Dirnagl, U., Iadecola, C., Moskowitz, M.A., 1999. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 22, 391–397.

Du, C., Fang, M., Li, Y., Li, L., Wang, X., 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102, 33–42.

Dubois-Dauphin, M., Frankowski, H., Tsujimoto, Y., Huarte, J., Martinou, J.C., 1994. Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. USA 91, 3309–3313.

Fiskum, G., Murphy, A.N., Beal, M.F., 1999. Mitochondria in neurodegeneration: acute ischemia and chronic neurogenerative disease. J. Cereb. Blood Flow Metab. 19, 351–369.

Foster, A.C., Gill, R., Wookruff, G.N., 1988. Neuroprotective effects of MK-801 in vivo: selectivity and evidence for delayed degeneration mediated by NMDA receptor activation. J. Neurosci. 8, 4745–4754.

Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halestrap, A.D., Wieloch, T., 1998. Cyclosporin A, but not FK-506, protects mitochondria and neurons against hypoglycemia damage and implicates the mitochondrial permeability transition. J. Neurosci. 18, 5151–5159.

Griffiths, E.J., Halestrap, A.P., 1991. Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. Biochem. J. 274, 611–614.

Halestrap, A.P., Davidson, A.M., 1990. Inhibition of Ca^{2+}-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem. J. 268, 153–160.

Kitagawa, K., Matsumoto, M., Tsujimoto, Y., Ohtsuki, T., Kuwabara, K., Matsushita, K., Yang, G., Tanabe, H., Martinou, J.C., Hori, M., Yanagihara, T., 1998. Amelioration of hippocampal neuronal damage after global ischemia by neuronal overexpression of Bcl-2 in transgenic mice. Stroke 29, 2616–2621.

Kowaltowski, A.J., Castilho, R.F., Vercesi, A.E., 2001. Mitochondrial permeability transition and oxidative stress. FEBS Lett. 495, 12–15.

Kowaltowski, A.J., Vercesi, A.E., Fiskum, G., 2000. Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. Cell Death Differ 7, 903–910.

Lafon-Cazal, M., Pietri, S., Culcasi, M., Bockaert, J., 1993. NMDA-dependent superoxide production and neurotoxicity. Nature 364, 535–537.

Leist, M., Nicotera, P., 1998. Calcium and neuronal death. Rev. Physiol. Biochem. Pharmacol. 132, 79–125.

Leventhal, L., Sortwell, C.E., Hanbury, R., Collier, T.J., Kordower, J.H., Palfi, S., 2000. Cyclosporin A protects striatal neurons in vitro and in vivo from 3-nitropropionic acid toxicity. J. Comp. Neurol. 425, 471–478.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c.. Cell 86, 147–157.

Maciel, E.N., Vercesi, A.E., Castilho, R.F., 2001. Oxidative stress in Ca^{2+}-induced membrane permeability transition in brain mitochondria. J. Neurochem. 79, 1237–1245.

Martinou, J.C., Dubois, -Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, M., Pietra, C., Huarte, J., 1994. Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13, 1017–1030.

Martinou, J.C., Green, D.R., 2001. Breaking the mitochondrial barrier. Nature Rev. Mol. Cell Biol. 2, 63–67.

Matsumoto, S., Friberg, H., Ferrand-Drake, M., Wieloch, T., 1999. Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion. J. Cereb. Blood Flow Metab. 19, 736–741.

Merad-Saidoune, M., Boitier, E., Nicole, A., Marsac, C., Martinou, J.C., Sola, B., Sinet, P.M., Ceballos-Picot, I., 1999. Overproduction of Cu/Zn-superoxide dismutase or Bcl-2 prevents the brain mitochondrial respiratory dysfunction induced by glutathione depletion. Exp. Neurol. 158, 428–436.

Murphy, A.N., Bredesen, D.E., Cortopassi, G., Wang, E., Fiskum, G., 1996. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc. Natl. Acad. Sci. USA 93, 9893–9898.

Nakao, N., Grasbon-Frodl, E.M., Widner, H., Brundin, P., 1996. Antioxidant treatment protects striatal neurons against excitotoxic insults. Neuroscience 73, 185–200.

Nicholls, D.G., Budd, S.L., 2000. Mitochondria and neuronal survival. Physiol. Rev. 80, 315–360.

Nicholls, D.G., Scott, I.D., 1980. The regulation of brain mitochondrial calcium-ion transport. The role of ATP in the discrimination between kinetic and membrane-potential- dependent calcium-ion efflux mechanisms. Biochem. J. 186, 833–839.

Nicolli, A., Basso, E., Petronilli, V., Wenger, R.M., Bernardi, P., 1996. Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. J. Biol. Chem. 271, 2185–2192.

Nieminen, A.L., Petrie, T.G., Lemasters, J.J., Selman, W.R., 1996. Cyclosporin A delays mitochondrial depolarization induced by N-methyl-D-aspartate in cortical neurons: evidence of the mitochondrial permeability transition. Neuroscience 75, 993–997.

Okonkwo, D.O., Povlishock, J.T., 1999. An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury. J. Cereb. Blood Flow Metab. 4, 443–451.

Orrenius, S., Ankarcrona, M., Nicotera, P., 1996. Mechanisms of calcium-related cell death. Adv. Neurol. 71, 137–149.

Petersen, A., Castilho, R.F., Hansson, O., Wieloch, T, Brundin, P., 2000. Oxidative stress, mitochondrial permeability transition and activation of caspases in calcium ionophore A23187-induced death of cultured striatal neurons. Brain Res. 857, 20–29.

Raghupathi, R., Fernandez, S.C., Murai, H., Trusko, S.P., Scott, R.W., Nishioka, W.K., McIntosh, T.K., 1998. Bcl-2 overexpression attenuates cortical cell loss after traumatic brain injury in transgenic mice. J. Cereb. Blood Flow Metab. 18, 1259–1269.

Reynolds, I.J., Hasting, T.G., 1995. Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. J. Neurosci. 15, 3318–3327.

Rosenthal, R.E., Hamud, F., Fiskum, G., Varghese, P.J., Sharpe, S., 1987. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. J. Cereb. Blood Flow Metab. 7, 752–758.

Rothman, S.M., Olney, J.W., 1986. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann. Neurol. 19, 105–111.

Rottenberg, H., Marbach, M., 1990. Regulation of Ca^{2+} transport in brain mitochondria. II. The mechanism of the adenine nucleotides enhancement of Ca^{2+} uptake and retention. Biochim. Biophys. Acta 1016, 87–98.

Schierle, G.S., Leist, M., Martinou, J.C., Widner, H., Nicotera, P., Brundin, P., 1999. Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. Eur. J. Neurosci. 11, 3073–3081.

Schinder, A.F., Olson, E.C., Spitzer, N.C., Montal, M., 1996. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J. Neurosci. 16, 6125–6133.

Schmued, L.C., Albertson, C., Slikker Jr., W., 1997. Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res. 751, 37–46.

Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H., Tsujimoto, Y., 1998. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc. Natl. Acad. Sci. USA 95, 1455–1459.

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., Kroemer, G., 1996. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184, 1331–1341.

Uchino, H., Elmer, E., Uchino, K., Li, P.A., He, Q.P., Smith, M.L., Siesjo, B.K., 1998. Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. Brain Res. 812, 216–226.

Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F., Reed, J.C., 1999. Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science 284, 339–343.

West, M.J., Slomianka, L., Gundersen, H.J., 1991. Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec. 231, 482–497.

Yang, J.C., Kahn, A., Cortopassi, G., 2000. Bcl-2 does not inhibit the permeability transition pore in mouse liver mitochondria. Toxicology 151, 65–72.

Yang, L., Matthews, R.T., Schulz, J.B., Klockgether, T., Liao, A.W., Martinou, J.C., Penney Jr, J.B., Hyman, B.T., Beal, M.F., 1998. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. J. Neurosci. 18, 8145–8152.

Zamzami, N., Marzo, I., Susin, S.A., Brenner, C., Larochette, N., Marchetti, P., Reed, J., Kofler, R., Kroemer, G., 1998. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. Oncogene 16, 1055–1063.

Zoratti, M., Szabòo, I., 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241, 139–176.

3.3-3°. TRABALHO

Maciel, E. N.; Kowaltowski, A. J.; Schwalm, F. D.; Rodrigues, J. M.; Souza, D.O.; Vercesi, A. E.; Wajner, M.; Castilho, R. F. Mitochondrial permeability transition in neuronal damage promoted by Ca^{2+} and respiratoty chain complex II inhibition.

Journal of Neurochemistry, versão re-submetida, 2004.

Mitochondrial permeability transition in neuronal damage promoted by Ca^{2+} and respiratory chain complex II inhibition

Evelise N. Maciel[1], Alicia J. Kowaltowski[2], Fábio D. Schwalm[3], Juliana M. Rodrigues[3], Diogo O. Souza[3], Anibal E. Vercesi[1], Moacir Wajner[3] and Roger F. Castilho[1]

[1]*Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP 13083-970, Brazil.* [2]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil.* [3]*Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brazil.*

Abstract

Changes in mitochondrial integrity, reactive oxygen species release and Ca^{2+} handling are proposed to be involved in the pathogenesis of many neurological disorders including methylmalonic acidemia and Huntington's disease, which exhibit partial mitochondrial respiratory inhibition. In this report, we studied the mechanisms by which the respiratory chain complex II inhibitors malonate, methylmalonate and 3-nitropropionate affect rat brain mitochondrial function and neuronal survival. All three compounds, at concentrations which inhibit respiration by 50%, induced mitochondrial inner membrane permeabilization when in the presence of micromolar Ca^{2+} concentrations. ADP, cyclosporin A and catalase prevented or delayed this effect, indicating it is mediated by reactive oxygen species and mitochondrial permeability transition (PT). PT induced by malonate, methylmalonate and 3-nitropropionate was also present in mitochondria isolated from liver and kidney, but required more significant respiratory inhibition. In brain, PT promoted by complex II inhibitors was stimulated by increasing Ca^{2+} cycling and absent when mitochondria were pre-loaded with Ca^{2+} or when Ca^{2+} uptake was prevented. In addition to isolated mitochondria, we determined the effect of methylmalonate on cultured PC12 cells and freshly prepared rat brain slices. Methylmalonate promoted cell death in striatal slices and PC12 cells, in a manner attenuated by cyclosporin A and bongkrekate, and unrelated to impairment of energy metabolism. We propose that under conditions in which mitochondrial complex II is partially inhibited in the CNS, neuronal cell death involves the induction of PT.

Keywords: energy metabolism, metabolic disease, calcium, free radicals, respiration, electron transport chain.

J. Neurochem. (resubmitted version)

Many neurological disorders are associated with mitochondrial electron transport chain inhibition, energy metabolism impairment and oxidative stress (for review, see Beal 1998; Nicholls and Budd 2000). Energy metabolism defects in neurons cause increases in intracellular Ca^{2+} levels, either by directly impairing Ca^{2+} removal systems or due to NMDA receptor activation (Albin and Greenamyre 1992; Fiskum *et al.* 1999; Nicholls and Budd 2000). During cytosolic Ca^{2+} overload, lytic enzymes such as phospholipase A_2, proteases and endonucleases

Address correspondence and reprint requests to Dr. Roger F. Castilho, Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP 13083-970, Brazil. Fax: +55 19 3788 9434; E-mail: roger@fcm.unicamp.br

Abbreviations used: BKA, bongkrekic acid; CsA, cyclosporin A; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone; MA, malonate; MMA, methylmalonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 3-NP, nitropropionate; PT, permeability transition; $\Delta\Psi$, mitochondrial transmembrane electrical potential.

are activated, resulting in cellular degradation (Orrenius *et al.* 2003). Under these conditions, the mitochondrion is the main organelle responsible for Ca^{2+} sequestration, a required step in NMDA-induced neurotoxicity (Budd and Nicholls 1996; Castilho *et al.* 1998; Stout *et al.* 1998). Excessive mitochondrial Ca^{2+} uptake and oxidative stress can cause non-selective inner mitochondrial membrane permeabilization, known as the permeability transition (PT) (Zoratti and Szabò 1995; Kowaltowski *et al.* 2001). PT results in mitochondrial Ca^{2+} release, organellar swelling, release of intramitochondrial apoptogenic factors such as cytochrome *c* (Liu *et al.* 1996) and loss of inner membrane potential and ATP synthesis. Since PT hampers oxidative phosphorylation and promotes the release of pro-apoptotic proteins, it can result both in necrosis and apoptosis (Kim *et al.* 2003). PT is inhibited by cyclosporin A (CsA), a drug shown to be protective in neuronal ischemia models (Nieminen *et al.* 1996; Schinder *et al.* 1996; Uchino *et al.* 1998; Petersen *et al.* 2000), suggesting the importance of PT in the pathogenesis of ischemic damage.

Changes in mitochondrial energy metabolism may also occur in other neurological disorders. In this work, we focused on respiratory chain complex II inhibition such as that observed in methylmalonic acidemia, a disorder of branched amino acid and odd-chain fatty acid metabolism, involving a defect in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A. This disease, which affects approximately 1 in 50,000 infants, is caused by a deficiency in methylmalonyl-CoA mutase activity. Patients present neurological manifestations such as seizure, encephalopathy and stroke, usually within the first months after birth (Matsui *et al.* 1983; Fenton *et al.* 2001). It is hypothesized that neurological damage in this disease is due to accumulation of methylmalonate (MMA) and inhibition of mitochondrial succinate dehydrogenase (complex II), resulting in impaired energy metabolism and oxidative stress (Dutra *et al.* 1993; Wajner and Coelho 1997; McLaughlin *et al.* 1998).

The concept that complex II inhibition and oxidative stress are a cause of neurological damage is supported by the finding that complex II inhibitors 3-nitropropionate (3-NP) and malonate (MA) can induce neurochemical, histological, and clinical features of another neurodegenerative disorder, Huntington's disease, in a mechanism sensitive to antioxidants (Beal *et al.* 1994; 1995). Interestingly, complex II/III is inhibited in the brains of Huntington's disease patients, in a manner most intense in the areas affected by the disease (Gu *et al.* 1996; Browne *et al.* 1997).

Based on the accumulating evidence that mitochondrial respiratory chain inhibition, oxidative stress and Ca^{2+} handling are involved in the pathogenesis of neurological diseases, we investigated the effects of complex II inhibitors on mitochondrial inner membrane integrity in the presence of Ca^{2+}.

Materials and methods

Animal care and use

The experimental protocols were approved by the Comittee for Ethics in Animal Research of the Universidade Estadual de Campinas in compliance with the Brazilian College for Animal Experimentation.

Isolation of rat forebrain mitochondria

Forebrain mitochondria were isolated as described by Rosenthal *et al* (1987) with minor modifications, from female Wistar rats weighing 200-250 g. Rats were killed by decapitation, their brains rapidly removed (within 1 min) and put into 10 ml of ice-cold "isolation buffer" containing 225 mM mannitol, 75 mM sucrose, 1 mM K^+-EGTA, 0.1% bovine serum albumin (BSA; free fatty acid), 10 mM K^+-HEPES pH 7.2 and 5 mg protease (P-8038, Sigma Chemical Co.). The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using both a loose fitting and a tight fitting pestle. The homogenate was centrifuged for 3 min at 2,000 g in a Beckman JA 20 rotor. After

centrifugation, the supernatant was recentrifuged for 8 min at 12,000 g. The pellet was resuspended in 20 ml "isolation buffer" containing 80 µl of 10% digitonin and recentrifuged for 8 min at 12,000 g. The supernatant was discarded and the final pellet gently washed and resuspended in "isolation buffer" devoid of EGTA, at an approximate protein concentration of 30-40 mg/ml. The respiratory control ratio (state 3/state 4 respiratory rate) was over 7.0, measured using succinate as a substrate. State 3 respiratory rates in the presence of succinate were in the range of 65-75 nmol O_2/min/mg.

Isolation of rat liver and kidney mitochondria
Rat liver and kidney mitochondria were isolated by the same procedure described above for forebrain mitochondria, including the addition of digitonin to the second pellet. Respiratory control ratios were above 5 using succinate.

Standard incubation procedure
The experiments using isolated mitochondria were carried out at 28°C, with continuous magnetic stirring, in a standard reaction medium containing 100 mM sucrose, 65 mM KCl, 10 mM K^+-HEPES buffer (pH 7.2), 30 µM EGTA, ~10 µM contaminating Ca^{2+}, 1 mM P_i and 5 mM succinate. Other additions are indicated in the figure legends. The results shown are representative of a series of at least four experiments using different mitochondrial preparations. The results were reproduced within 10% variation.

Oxygen uptake measurements
Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, OH, USA) in 1.3 ml of standard reaction medium (28°C), in a sealed glass cuvette equipped with a magnetic stirrer. Oxygen uptake measurements were conducted in standard reaction medium containing 200 µM EGTA.

Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)
Mitochondrial $\Delta\Psi$ was estimated through fluorescence changes of safranin O (5 µM) recorded on a model F-4010 Hitachi spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm (Åkerman and Wikström 1976). Using this technique, the initial fluorescence change promoted by Ca^{2+} uptake appears less pronounced than the response using tetraphenylphosphonium electrodes due to the non-logarithmic relationship to $\Delta\Psi$ using safranin, and a possible slower distribution of safranin between the mitochondrial and buffer fractions.

Determination of mitochondrial swelling
Mitochondrial swelling was estimated through light scattering changes recorded on a spectrofluorometer operating at excitation and emission wavelengths of 520 nm, with slit widths of 1.5 nm (Andreyev *et al.* 1998).

Determination of NAD(P) redox state
The oxidation or reduction of endogenous pyridine nucleotides in the mitochondrial suspension was followed by measuring fluorescence levels at excitation and emission lengths of 366 and 450 nm, respectively.

PC12 cell cultures
Cultured pheochromocytoma cells (PC12 cells) were continuously maintained at 37°C and 5% CO_2 in Modified Eagle's Media (4.5 g/l glucose) supplemented with 10% horse serum and 5% fetal bovine serum. MMA (20 mM) treatments were conducted in the same media, during 24 h. Medium pH after incubation with MMA was measured using a conventional pH electrode.

Cellular content and viability
Cell content and viability were measured by evaluating DNA-bound ethidium bromide fluorescence (excitation = 365 nm, emission = 580 nm) before and after the addition of 0.1% digitonin. In the absence of digitonin, ethidium bromide fluorescence is proportional to the number of dead cells with compromised plasma membranes. Fluorescence in the presence digitonin, at concentrations sufficient to permeabilize the plasma membrane to ethidium bromide, is proportional to

the total number of cells (Karsten 1980), as verified by parallel cell counts (not shown). Since PC12 cells present doubling times of approximately 72 hours and MMA treatments are conducted during 24 hours, differences in cell content probably reflect an inhibition of PC12 proliferation in addition to cell damage and death.

Brain slice preparation

Rats were killed by cervical dislocation, decapitated and the brain removed (Pull and MacIlwain 1972; Marshall *et al.* 1996). The striatal area was dissected and sliced coronally (400 μm) with a McIlwan tissue chopper. The slices were washed with 1 ml in Hanks' Balanced Salt Solution (HBSS) containing 1.26 mM $CaCl_2$, 0.41 mM $MgSO_4$ and 5.55 mM glucose and pre-incubated at 37°C with 300 μl of the same medium bubbled with 5% CO_2. The pH was maintained at 7.4. Under control conditions, these slices presented 70-80% viability just after isolation and 60-70% viability after 8 h. The initial 20-30% viability decrease is most probably caused by mechanical damage.

MTT reduction

Neuronal injury in brain slices was quantified by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a dark violet formazan product (Mosmann 1983). After incubation, the slices were washed twice with 1 ml in HBSS. MTT reduction assays were performed in plates containing 300 μl HBSS, and the reaction was started by adding 0.5 mg/ml MTT. After 45 min incubation at 37°C, medium was removed and the slices dissolved in DMSO. The rate of MTT reduction was measured spectrophotometrically at a test wavelength of 570 nm and a reference wavelength of 630 nm. In intact cells, MTT reduction is virtually unaffected by mitochondrial succinate dehydrogenase (Liu *et al.* 1997).

Statistical analysis

Data in Figs. 9 and 10 were analyzed by multiple pair-wise comparison Tukey tests performed by SigmaStat, and are presented as averages ± standard errors of at least 3 experiments conducted with different preparations. All other figures depict representative traces of ≥ 3 repetitions, except Figs. 1A-C, 5A-B and 6A-B, which present averages ± standard errors of at least 3 experiments.

Chemicals

Most chemicals, including A23187, alamethicin, antimycin A, digitonin, FCCP, malonic acid, methylmalonic acid, 3-nitropopionic acid, protease type VIII, rotenone, safranine O, malic acid, pyruvic acid, succinic acid and MTT were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A). MA, MMA and 3-NP were prepared by dissolving the respective acids in water and adjusting the pH to 7.2 with KOH.

Results

In order to test the effects of complex II inhibition on brain mitochondrial integrity and increase our understanding of neuronal injury occurring in methylmalonic acidemia and Huntington's disease, we initially determined the concentrations of MA, MMA and 3-NP which inhibit succinate-supported respiratory activity by 50% (Fig. 1 A-C). We found that 40 μM MA, 4 mM MMA and 300 μM 3-NP lead to approximately 50% inhibition of intact isolated brain mitochondrial succinate-supported respiration stimulated by oxidative phosphorylation. This inhibition is insufficient to significantly decrease the mitochondrial inner membrane potential ($\Delta\Psi$) in the absence oxidative phosphorylation and Ca^{2+} (Fig. 1 D-F, *lines b*). However, in the presence of either MA, MMA or 3-NP, $\Delta\Psi$ is dissipated if a 40 μM Ca^{2+} addition is made (*lines e*). This dissipation is most intense using MA (Panel D), but also occurs within a few minutes in the presence of MMA (Panel E) and 3-NP (Panel F). Qualitatively similar results were obtained using MA when experiments were conducted at 37°C. Only a slight $\Delta\Psi$ decrease is induced by Ca^{2+} alone (*lines d*). In all cases, $\Delta\Psi$ reached very low levels, and the further addition of the proton ionophore FCCP changed fluorescence levels only slightly. The loss of $\Delta\Psi$ was prevented by the concomitant presence of CsA and ADP (*lines c*), potent PT

inhibitors in brain (Brustovetsky and Dubinsky 2000; Maciel *et al.* 2001; 2003), suggesting it is not directly related to respiratory inhibition or Ca^{2+} uptake, but their additive effect on inner mitochondrial membrane integrity. In order to confirm that the $\Delta\Psi$ decrease observed in Fig. 1 was indeed related to non-selective inner membrane permeabilization due to PT, we measu-red mitochondrial swelling as a consequence of ion, sucrose and water uptake, following light scattering of the mitochondrial suspension (Fig.2). Alamethicin, which forms large pores in the inner mitochondrial membrane, was added at the end of each trace to cause maximal swelling. We found that MA (Panel A), MMA (Panel B) or 3-NP (Panel C) and Ca^{2+} caused extensive mitochondrial swelling (*lines e*). This swelling was not observed in the presence of the respiratory inhibitors (*lines b*) or Ca^{2+} (*lines d*) alone. Confirming that this swelling was due to PT, CsA plus ADP fully prevented the light scattering changes observed (*lines c*).

Catalase, which was previously shown to prevent PT by removing mitochondrial H_2O_2 (Valle *et al.* 1993; Castilho *et al.* 1995; Kowaltowski *et al.* 1996; Maciel *et al.* 2001), was also tested on $\Delta\Psi$ decreases promoted by Ca^{2+} and complex II inhibition (Fig. 3). We found that catalase partially prevented and delayed the loss of $\Delta\Psi$ (compare *lines c* and *d*), suggesting that inner mitochondrial membrane permeabilization under these conditions is at least partially caused by the accumulation of mitochondrial H_2O_2.

In Fig. 4A, NADH-linked substrates were added to the reaction medium to verify their effect on MA and Ca^{2+}-induced $\Delta\Psi$ dissipation and investigate if the observed effect was due to the lack of respiratory substrates to support the generation of $\Delta\Psi$. We found that NADH-linked substrates α-ketoglutarate, glutamate, malate and pyruvate (*line c*) partially prevent $\Delta\Psi$ dissipation induced by Ca^{2+} and MA (*line d*). Interestingly, this prevention was stimulated by the presence of the complex I inhibitor rotenone (*line b*), a condition in which NADH-linked substrates cannot support $\Delta\Psi$.

Parallel measurements of mitochondrial NAD(P)H fluorescence (Fig. 4B) indicate that pyridine nucleotide reduction is increased by rotenone and NADH-linked substrates (*line a*), confirming that PT is inhibited when NAD(P)H is reduced. High NADH levels such as those occurring in the presence of rotenone have been linked to prevention of PT by promoting $NADP^+$ reduction catalyzed by the mitochondrial transhydrogenase (Lehninger *et al.* 1978; Hoek and Rydstrom 1988; Zago *et al.* 2000; Kowaltowski *et al.* 2001), improving mitochondrial redox capacity (Le-Quoc and Le-Quoc 1989; Kehrer and Lund 1994). Thus, our finding that NADH-linked substrates and rotenone prevent MA and Ca^{2+}-induced $\Delta\Psi$ loss further support the hypothesis that this loss is caused by mitochondrial oxidative stress. On the other hand, no oxidation of NAD(P)H is observed when MA and Ca^{2+} are added under conditions in which PT is inhibited (*line b*), suggesting that NAD(P)H oxidation by complex II inhibitors and Ca^{2+} is not the cause of PT under these conditions.

Next, we investigated if the induction of PT was specific for complex II inhibitors in the presence of Ca^{2+} or could also be observed with partial inhibitions of other respiratory complexes (Fig. 5). Antimycin A (a complex III inhibitor) and rotenone (a complex I inhibitor) were added to mitochondrial suspensions at a concentration that inhibited maximized respiration by 50% (Panels A and B). At these concentrations, antimycin and rotenone did not cause a large $\Delta\Psi$ decrease after the addition of Ca^{2+} (Panels C and D, *lines d*). This result indicates that the $\Delta\Psi$ loss observed due to PT in the previous figures occurs exclusively in response to partial complex II inhibition.

Our next experiments tested the effects of partial complex II inhibition and Ca^{2+} on inner membrane integrity of mitochondria isolated from liver and kidney (Fig. 6), in order to establish if the $\Delta\Psi$ decrease was specific for brain. MA was used as a complex II inhibitor since it was found to be the most potent PT inducer (Fig. 1). MA concentrations required to inhibit respiration by 50% in liver and kidney mitochondria were similar to those in brain (Panels A and B). However, these MA concentrations only slightly increased (*lines d*) the $\Delta\Psi$ drop induced by 100 μM Ca^{2+} alone (*lines c*). Higher MA concentrations, resulting in more

significant (60-80%) respiratory inhibition, did promote a $\Delta\Psi$ decrease (*lines e* and *f*), but still required incubation times much longer than in brain. Thus, brain mitochondria seem to be more sensitive to PT induced by Ca^{2+} and partial complex II inhibition.

In order to gain insight into the mechanism through which complex II inhibition and Ca^{2+} lead to PT, the role of mitochondrial Ca^{2+} uptake was investigated (Fig. 7). MMA was used in these experiments since it probably has a more important pathological role than MA or 3-NP. We found that the $\Delta\Psi$ decrease promoted by MMA and Ca^{2+} (*line d*) was much less intense when mitochondria were allowed to take up Ca^{2+} before respiratory inhibition by MMA (*line b*). This suggests that mitochondrial Ca^{2+} uptake under conditions of partial complex II inhibition is an underlying mechanism of PT induction. In order to confirm this hypothesis, we tested the effect of Na^+ ions, which increase mitochondrial Ca^{2+} cycling (Crompton *et al.* 1978). As expected, Na^+ ions lead to increased $\Delta\Psi$ loss (*lines c* and *e*, compare with *lines b* and *d*, respectively). On the other hand, the inhibitor of the Ca^{2+} uniporter ruthenium red (*line a*) fully prevented $\Delta\Psi$ loss caused by MMA in Ca^{2+}-loaded mitochondria treated with Na^+.

The effect of Ca^{2+} uptake on MMA-induced PT was also investigated in mitochondria treated with the proton ionophore FCCP to prevent active Ca^{2+} accumulation (Fig. 8). The Ca^{2+} ionophore A23187 was added to ensure free Ca^{2+} transport across the mitochondrial membranes. Under these conditions, swelling promoted by 200 μM Ca^{2+} alone (*line c*) was not increased by MMA (*line d*), although subsequent Ca^{2+} and alamethicin additions indicate that swelling could still occur.

Next, we were interested in determining if PT induced by partial complex II inhibition occurred within intact cells, and verifying the effects of this process on cell survival. Again, MMA was used due to its potential pathological relevance (Matsui *et al.* 1983; Fenton *et al.* 2001). In Fig. 9, PC12 cells were cultured in the presence of MMA for 24 h. This resulted in a statistically significant decrease in cell content (Panel A, *empty bars*) and an increase in cell death (*gray bars*). Both the loss of cell viability and content were fully recovered by the concomitant presence of CsA, indicating this effect is caused by PT. Indeed, the cells were also protected by bongkrekic acid (BKA), which inhibits the mitochondrial ADP/ATP translocator and prevents PT (Vercesi 1984; Zoratti and Szabo 1995; Zamzami *et al.* 1996). FK506, which presents similar calcineurin-inhibitory effects to CsA but does not prevent PT (Griffiths and Halestrap 1991; Friberg *et al.* 1998; Uchino *et al.* 2002), did not protect against MMA-induced damage in PC12 cells when tested at various concentrations (0.01 – 1 μM). The higher concentrations of FK506 resulted in extensive cell death in the absence of MMA (not shown).

In addition to promoting cell death, MMA significantly decreased medium pH (Panel B). This effect was not due to the presence of MMA itself, since it was prevented by CsA. Medium acidification might be due to anaerobic glycolysis in cells unable to adequately reoxidize NADH due to PT and cytochrome *c* loss (Huang *et al.* 2001; Schild *et al.* 2003). Indeed, BKA, which inhibits the respiratory chain by avoiding ADP transport into mitochondria, also promoted medium acidification. Acidification by BKA was not observed prior to 24 h incubation with MMA, indicating it is not related to the acidity of the drug itself. The finding that BKA prevents cell damage but not medium acidification indicates that the pH decrease promoted by MMA is not responsible for the increase in cell death and decreased cell content.

The effect of MMA was also tested on freshly prepared rat striatal slices (Pull and MacIlwain 1972; Marshall *et al.* 1996), in order to ensure that it is valid for adult, non-proliferating, neuronal cells (Fig. 10). Lactate dehydrogenase release measurements to estimate cell damage were not attainable, since this enzyme was directly inhibited by MMA (results not shown). Slice viability was measured by estimating MTT reduction, which was not affected by antimycin A and, therefore, is not directly related to respiratory inhibition. Various MMA exposure times and concentrations were tested, and the concentration which inhibited slice viability by ~50% (3 mM) at 3 hours was chosen to conduct further studies on slice viability. The

decrease in slice viability promoted by MMA under these conditions was prevented by CsA, confirming that this loss of viability is related to PT. In addition, antimycin A did not decrease slice viability, indicating that respiratory inhibition alone does not lead to cell death under these conditions.

Discussion

To understand the consequences of partial complex II inhibition and its influence on CNS mitochondrial PT, we monitored the effects of chemically induced complex II inhibition on isolated brain mitochondria. Both competitive (MA) and noncompetitive and irreversible (3-NP) respiratory inhibitors were used. MMA, which inhibits respiration by producing intra-mitochondrial metabolites (Okun *et al.* 2002; Kolker *et al.* 2003) was also tested due to its pathological relevance in methylmalonic acidemia (Matsui *et al.* 1983; Fenton *et al.* 2001). We found that ~50% inhibition of ADP-stimulated mitochondrial respiration by MA, MMA or 3-NP had a synergistic effect with Ca^{2+}, leading to extensive $\Delta\Psi$ loss. This $\Delta\Psi$ loss was caused by mitochondrial PT, since it was accompanied by swelling and could be prevented by cyclosporin A plus ADP. Interestingly, MA-induced PT was much less prominent in liver and kidney mitochondria. This finding brings further support to work describing different characteristics of PT and mitochondrial Ca^{2+} handling in brain (Andreyev and Fiskum 1999; Berman *et al.* 2000; Brustovetsky *et al.* 2003), and may be due to intrinsic differences in respiratory chain function and electron leakage between these mitochondrial. These data are also in line with the finding that CNS damage prevails under pathological conditions in which complex II is chemically inhibited exogenously or due to metabolic deficiencies such as methylmalonic acidemia (Matsui *et al.* 1983; Brouillet *et al.* 1993; Roodhooft *et al.* 1990; Wajner and Coelho 1997; Gabrielson *et al.* 2001).

$\Delta\Psi$ loss and swelling in brain mitochondria were also partially prevented by catalase, which removes mitochondrial H_2O_2, indicating that PT under these conditions is probably associated with mitochondrial oxidative stress, as observed in many models (see Kowaltowski *et al.* 2001 for review). We also found that PT promoted by MMA plus Ca^{2+} is prevented by NADH-linked substrates in the presence of rotenone, a condition in which these substrates cannot support $\Delta\Psi$. These results indicate that PT promoted by MMA is prevented by the accumulation of intramitochondrial NADH, increasing mitochondrial redox capacity (Le-Quoc and Le-Quoc 1989; Kehrer and Lund 1994; Kowaltowski *et al.* 2001). Our finding is in line with many previous results showing inhibition of PT by mitochondrial NADH and NADPH accumulation (Lehninger *et al.* 1978; Hoek and Rydstrom 1988; Zago *et al.* 2000). However, even though we found that the maintenance of reduced NAD(P)H can protect against Ca^{2+} and complex II inhibition-stimulated PT, we did not find a decrease in NAD(P)H levels in the presence of CsA, suggesting this is not the cause of oxidative stress under these conditions (Fig. 4B).

Mitochondrial reactive oxygen release is strongly increased in the presence of respiratory inhibitors, in a manner which may be stimulated by Ca^{2+}. As an example, complex I inhibition and Ca^{2+} accumulation increase reactive oxygen species release (Starkov *et al.* 2002; Sousa *et al.* 2003). However, this respiratory inhibition-induced oxidative stress is not necessarily a cause of PT, as indicated by the lack of $\Delta\Psi$ loss when Ca^{2+} and rotenone or antimycin A were added together. Thus, under our experimental conditions, respiratory inhibition and/or oxidative stress alone are not sufficient to cause PT.

The cause for increased susceptibility to PT seems to involve the lack of ability to respond adequately with respiratory increases upon Ca^{2+} uptake when succinate-sustained respiration is partially inhibited. Indeed, stimulating Ca^{2+} cycling through the Ca^{2+}/Na^{+} exchanger by adding extramitochondrial Na^{+} intensifies the MMA effect. PT is also less prominent when mitochondria are allowed to accumulate Ca^{2+} before MMA is added. In addition, MMA-induced PT is not observed in uncoupled mitochondria in which passive Ca^{2+} uptake is ensured by the presence of A23187. Thus, it is probable that under conditions in which

respiration is partially inhibited by MMA, the increased electron transport necessity incurred by Ca^{2+} uptake leads to PT. This suggests that decreases of succinate dehydrogenase electron transport rates lead mitochondria to a threshold in which sensitivity to endogenous oxidants increases upon $\Delta\Psi$-driven Ca^{2+} uptake.

The occurrence of PT in intact cells in a model of methylmalonic acidemia was confirmed through the finding that CsA prevented cultured PC12 cell death induced by MMA. CsA also prevented MMA-induced cell death in adult, non-proliferating, striatal slices. BKA, which inhibits PT by binding to the adenine nucleotide translocator, a presumed component of the PT pore (Beutner *et al.* 1996; Marzo *et al.* 1998; Haworth and Hunter 2000), prevented cell death induced by MMA. Since BKA inhibits oxidative phosphorylation, the maintenance of cell viability in its presence indicates that the main cytotoxic effect of MMA is not the impairment of energy metabolism in PC12 cells, which are highly glycolitic. Indeed, incubation media in the presence of BKA were significantly acidified compared to controls, suggesting these cells were more actively glycolytic and generated more lactate. In addition, the respiratory inhibitor antimycin A was ineffective in leading to PT and decreasing striatal slice viability. Thus, cell death appears to be caused by non-selective inner membrane permeabilization due to PT, and not by direct metabolic effects of MMA.

In conclusion, our findings indicate that PT is a cause of the loss of neural viability induced by complex II inhibition. These results are in line with studies showing increased susceptibility of striatal mitochondria to Ca^{2+}-induced PT (Brustovetsky *et al.* 2003), and that CsA protects against 3-NP toxicity to striatal neurons (Leventhal *et al.* 2000). Thus, we suggest the possibility that PT inhibitors may be interesting therapeutic candidates for the prevention of neuronal damage under these conditions, and should be thoroughly investigated using *in vivo* models of neurotoxicity in methylmalonic acidemia and Huntington's disease.

Acknowledgments: The authors thank Dr. Marcus Frizzo (Federal University of Rio Grande do Sul, Porto Alegre) for valuable discussions and Edilene Siqueira and Edson A. Gomes for technical support. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Programa de Apoio à Núcleos de Excelência (PRONEX) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). E.N.M. is a student supported by FAPESP.

References

Åkerman K. E. and Wikström M. K. (1976) Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett.* **68,** 191-197.

Albin R. L. and Greenamyre J. T. (1992) Alternative excitotoxic hypotheses. *Neurology* **42,** 733-738.

Andreyev A. Y., Fahy B. and Fiskum G. (1998) Cytochrome *c* release from brain mitochondria is independent of the mitochondrial permeability transition. *FEBS Lett.* **439,** 373-376.

Beal M. F. (1998) Mitochondrial dysfunction in neurodegenerative diseases. *Biochim. Biophys. Acta* **1366,** 211-223.

Beal M. F., Ferrante R. J., Henshaw R., Matthews R. T., Chan P. H., Kowall N. W., Epstein C. J. and Schulz J. B. (1995) 3-Nitropropionic acid neurotoxicity is attenuated in copper/zinc superoxide dismutase transgenic mice. *J. Neurochem.* **65,** 919-922.

Beal M. F., Henshaw D. R., Jenkins B. G., Rosen B. R. and Schulz J. B. (1994) Coenzyme Q10 and nicotinamide block striatal lesions produced by the mitochondrial toxin malonate. *Ann. Neurol.* **36,** 882-888.

Berman S. B., Watkins S. C. and Hastings T. G. (2000) Quantitative biochemical and ultrastructural comparison of mitochondrial permeability transition in isolated brain and liver mitochondria: evidence for reduced sensitivity of brain mitochondria. *Exp. Neurol.* **164,** 415-425.

Beutner G., Ruck A., Riede B., Welte W. and Brdiczka D. (1996) Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. *FEBS Lett.* **396,** 189-195.

Browne S. E., Bowling A. C., MacGarvey U., Baik M. J., Berger S. C., Muqit M. M., Bird E. D. and Beal M. F. (1997) Oxidative damage and metabolic dysfunction

in Huntington's disease: selective vulnerability of the basal ganglia. *Ann. Neurol.* **41,** 646-653.

Brouillet E., Jenkins B. G., Hyman B. T., Ferrante R. J., Kowall N. W., Srivastava R., Roy D. S., Rosen B.R. and Beal M. F. (1993) Age-dependent vulnerability of the striatum to the mitochondrial toxin 3-nitropropionic acid. *J. Neurochem.* **60,** 356-359.

Brustovetsky N. and Dubinsky J. M. (2000) Dual responses of CNS mitochondria to elevated calcium. *J. Neurosci.* **20,** 103-113.

Brustovetsky N., Brustovetsky T., Purl K. J., Capano M., Crompton M. and Dubinsky J. M. (2003) Increased susceptibility of striatal mitochondria to calcium-induced permeability transition. *J. Neurosci.* **23,** 4858-4867.

Budd S. L. and Nicholls D. G. (1996) Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurochem.* **67,** 2282-2291.

Castilho R. F., Kowaltowski A. J., Meinnicke A. R., Bechara E. J. and Vercesi A. E. (1995) Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. *Free Radic. Biol. Med.* **18,** 479-486.

Castilho R. F., Hansson O., Ward M. W., Budd S. L. and Nicholls D. G. (1998) Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurosci.* **18,** 10277-10286.

Crompton M., Moser R., Ludi H. and Carafoli E. (1978) The interrelations between of the transport of sodium and calcium in mitochondria of various mammalian tissues. *Eur. J. Biochem.* **82,** 25-31.

Dutra J. C., Dutra-Filho C. S., Cardozo S. E., Wannmacher C. M., Sarkis J. J. and Wajner M. (1993) Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. *J. Inherit. Metab. Dis.* **16,** 147-153.

Fenton W. A., Gravel R. A. and Rosenblatt D. S. (2001) Disorders of propionate and methylmalonate metabolism. In: The Metabolic and Molecular Bases of Inherited Disease (Scriver C. R., Beaudet A. L., Sly W. S. and Valle D., eds.), 8th edition, pp. 2165-2193. New York: McGraw-Hill.

Fiskum G., Murphy A. N. and Beal M. F. (1999) Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenenerative disease. *J. Cereb. Blood Flow Metab.* **19,** 351-369.

Friberg H., Ferrand-Drake M., Bengtsson F., Halestrap A. D. and Wieloch T. (1998) Cyclosporin A, but not FK-506, protects mitochondria and neurons against hypoglycemia damage and implicates the mitochondrial permeability transition. *J. Neurosci.* **18,** 5151-5159.

Gabrielson K. L., Hogue B. A., Bohr V. A., Cardounel A. J., Nakajima W., Kofler J., Zweier J. L., Rodriguez E. R., Martin L. J., de Souza-Pinto N.C. and Bressler J. (2001) Mitochondrial toxin 3-nitropropionic acid induces cardiac and neurotoxicity differentially in mice. *Am. J. Pathol.* **159,** 1507-1520.

Griffiths E. J. and Halestrap A. P. (1991) Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. *Biochem. J.* **274,** 611-614.

Gu M., Gash M. T., Mann V. M., Javoy-Agid F., Cooper J. M. and Schapira A. H. (1996) Mitochondrial defect in Huntington's disease caudate nucleus. *Ann. Neurol.* **39,** 385-389.

Haworth R. A. and Hunter D. R. (2000) Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria. *J. Bioenerg. Biomembr.* **32,** 91-96.

Hoek J. B. and Rydström J. (1988) Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem. J.* **254,** 1-10.

Huang X., Zhai D. and Huang Y. (2001) Dependence of permeability transition pore opening and cytochrome *c* release from mitochondria on mitochondria energetic status. *Mol. Cell. Biochem.* **224,** 1-7.

Karsten U. (1980) Fluorometric estimation of dead cells in cell suspensions. *Experientia* **36,** 263-264.

Kehrer J. P. and Lund L. G. (1994) Cellular reducing equivalents and oxidative stress. *Free Radic. Biol. Med.* **17,** 65-75.

Kim J. S., He L. and Lemasters J. J. (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. *Biochem. Biophys. Res. Commun.* **304,** 463-470.

Kolker S., Schwab M. A., Horster F., Sauer S., Hinz A., Wolf N. I., Mayatepek E., Hoffmann G. F., Smeitink J. A. and Okun J. G. (2003) Methylmalonic acid: Biochemical hallmark of methylmalonic acidurias but no inhibitor of mitochondrial respiratory chain. *J. Biol. Chem.* **278,** 47388-47393.

Kowaltowski A. J., Castilho R. F. and Vercesi A. E. (1996) Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species. *FEBS Lett.* **378,** 150-152.

Kowaltowski A. J., Castilho R. F. and Vercesi A. E. (2001) Mitochondrial permeability transition and oxidative stress. *FEBS Lett.* **495,** 12-15.

Lehninger A. L., Vercesi A. and Bababunmi E. A. (1978) Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides. *Proc. Natl. Acad. Sci. USA* **75,** 1690-1694.

Le-Quoc D. and Le-Quoc K. (1989) Relationships between the NAD(P) redox state, fatty acid oxidation, and inner membrane permeability in rat liver mitochondria. *Arch. Biochem. Biophys.* **273,** 466-478.

Leventhal L., Sortwell C.E., Hanbury R., Collier T.J., Kordower J.H. and Palfi S. (2000) Cyclosporin A protects striatal neurons in vitro and in vivo from 3-nitropropionic acid toxicity. *J. Comp. Neurol.* **425,** 471-478.

Liu X., Kim C.N., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. *Cell* **86,** 147-157.

Liu Y., Peterson D. A., Kimura H. and Schubert D. (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* **69,** 581-593.

Maciel E. N., Kaminski Schierle G. S., Hansson O., Brundin P., Castilho R. F. (2003) Cyclosporin A and Bcl-2 do not inhibit quinolinic acid-induced striatal excitotoxicity in rodents. *Exp Neurol* **183,** 430-437.

Maciel E. N., Vercesi A. E. and Castilho R. F. (2001) Oxidative stress in Ca^{2+}-induced membrane permeability transition in brain mitochondria. *J. Neurochem.* **79,** 1237-1245.

Marshall D., Soliakov L., Redfern P. and Wonnacott S. (1996) Tetrodoxin-sensitivity of nicotine-evoked dopamine release from rat striatum. *Neuropharmacol.* **35,** 1531-1536.

Marzo I., Brenner C., Zamzami N., Susin S. A., Beutner G., Brdiczka D., Remy R., Xie Z. H., Reed J. C. and Kroemer G. (1998) The permeability transition pore complex: a target for apoptosis regulation by caspases and bcl-2-related proteins. *J. Exp. Med.* **187,** 1261-1271.

Matsui S. M., Mahoney M. J. and Rosenberg L. E. (1983) The natural history of the inherited methylmalonic acidemias. *N. Engl. J. Med.* **308,** 857-861.

McLaughlin B. A., Nelson D., Silver I. A., Erecinska M. and Chesselet M. F. (1998) Methylmalonate toxicity in primary neuronal cultures. *Neuroscience* **86,** 279-290.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods* **16,** 55-63.

Nicholls D. G. and Budd S. L. (2000) Mitochondria and neuronal survival. *Physiol. Rev.* **80,** 315-360.

Nieminen A. L., Petrie T. G., Lemasters J. J. and Selman W. R. (1996) Cyclosporin A delays mitochondrial depolarization induced by *N*-methyl-D-aspartate in cortical neurons: evidence of the mitochondrial permeability transition. *Neuroscience* **75,** 993-997.

Okun J. G., Horster F., Farkas L. M., Feyh P., Hinz A., Sauer S., Hoffmann G. F., Unsicker K., Mayatepek E. and Kolker S. (2002) Neurodegeneration in methylmalonic aciduria involves inhibition of complex II and the tricarboxylic acid cycle, and synergistically acting excitotoxicity. *J. Biol. Chem.* **277,** 14674-14680.

Orrenius S., Zhivotovsky B. and Nicotera P. (2003) Regulation of cell death: the calcium-apoptosis link. *Nat. Rev. Mol. Cell. Biol.* **4,** 552-565.

Petersen A., Castilho R.F., Hansson O., Wieloch T. and Brundin P. (2000) Oxidative stress, mitochondrial permeability transition and activation of caspases in calcium ionophore A23187-induced death of cultured striatal neurons. *Brain Res.* **857,** 20-29.

Pull I., McIlwain H. (1972) Metabolism of (^{14}C)adenine and derivatives by cerebral tissues, superfused and electrically stimulated. *Biochem. J.* **126,** 965-973.

Roodhooft A. M., Baumgartner E. R., Martin J. J., Blom W. and Van Acker K. J. (1990) Symmetrical necrosis of the basal ganglia in methylmalonic acidaemia. *Eur. J. Pediatr.* **149,** 582-584.

Rosenthal R. E., Hamud F., Fiskum G., Varghese P. J. and Sharpe S. (1987) Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J. Cereb. Blood Flow Metab.* **7,** 752-758.

Schild L., Huppelsberg J., Kahlert S., Keilhoff G. and Reiser G. (2003) Brain mitochondria are primed by moderate Ca^{2+} rise upon hypoxia/reoxygenation for functional breakdown and morphological disintegration. *J. Biol. Chem.* **278,** 25454-25460.

Schinder A. F., Olson E. C., Spitzer N. C. and Montal M. (1996) Mitochondrial dysfunction is a primary event

in glutamate neurotoxicity. *J. Neurosci.* **16,** 6125-6133.

Sousa S. C., Maciel E. N., Vercesi A. E. and Castilho R. F. (2003) Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. *FEBS Lett.* **543,** 179-183.

Starkov A. A., Polster B. M. and Fiskum G. (2002) Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax. *J. Neurochem.* **83,** 220-228.

Stout A. K., Raphael H. M., Kanterewicz B. I., Klann E. and Reynolds I. J. (1998) Glutamate-induced neuron death requires mitochondrial calcium uptake. *Nat. Neurosci.* **1,** 366-373.

Uchino H., Elmer E., Uchino K., Li P. A., He Q. P., Smith M.L. and Siesjo B. K. (1998) Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. *Brain Res.* **812,** 216-226.

Uchino H., Minamikawa-Tachino R., Kristián T., Perkins G., Narazaki M., Siesjö B. K. and Shibasaki F. (2002) Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. *Neurobiol. Dis.* **10,** 219-233.

Valle V., Fagian M. M., Parentoni L. S., Meinicke A. R. and Vercesi A. E. (1993) The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants. *Arch. Biochem. Biophys.* **252,** 171-178.

Vercesi A. E. (1984) Possible participation of membrane thiol groups on the mechanism of $NAD(P)^+$-stimulated Ca^{2+} efflux from mitochondria. *Biochem. Biophys. Res. Commun.* **119,** 305-310.

Wajner M. and Coelho J. C. (1997) Neurological dysfunction in methylmalonic acidaemia is probably related to the inhibitory effect of methylmalonate on brain energy production. *J. Inherit. Metab. Dis.* **20,** 761-768.

Zago E. B., Castilho R. F. and Vercesi A. E. (2000) The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. *FEBS Lett.* **478,** 29-33.

Zamzami N., Marchetti P., Castedo M., Hirsch T., Susin S. A., Masse B. and Kroemer G. (1996) Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett.* **384,** 53-57.

Zoratti M. and Szabò I. (1995) The mitochondrial permeability transition. *Biochim. Biophys. Acta* **1241,** 139-176.

Legends to Figures

Fig. 1 - Partial inhibition of mitochondrial respiratory chain complex II induces transmembrane electrical potential ($\Delta\Psi$) dissipation in the presence of Ca^{2+}. Isolated rat forebrain mitochondria (BM) (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O. **Panels A-C**, mitochondrial oxygen consumption measurements in the presence of 1 mM ADP and varying concentrations of respiratory chain complex II inhibitors malonate (MA) (**Panel A**), methylmalonate (MMA) (**Panel B**) or 3-nitropropionate (3-NP) (**Panel C**). **Panels D-F**, mitochondrial transmembrane electrical potential ($\Delta\Psi$) measurements. 40 μM MA (**Panel D**, *lines b, c, e*), 4 mM MMA (**Panel E**, *lines b, c, e*) or 300 μM 3-NP (**Panel F**, *lines b, c, e*) were added where indicated. Ca^{2+} (40 μM) was added to the experiments represented by the *lines c, d, e*. *Lines c* represent experiments conducted in the presence of the mitochondrial permeability transition (PT) inhibitors 1 μM cyclosporin A, 200 μM ADP and 1 μg/ml oligomycin. *Lines a* represent control experiments in the absence of respiratory inhibitors and Ca^{2+}. FCCP (1 μM) was added where indicated by the arrows. a.u. = Arbitrary units.

Fig. 2 - Partial inhibition of mitochondrial respiratory chain complex II induces mitochondrial swelling in the presence of Ca^{2+}. BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium. 40 μM MA (**Panel A**, *lines b, c, e*), 4 mM MMA (**Panel B**, *lines b, c, e*) or 300 μM 3-NP (**Panel C**, *lines b, c, e*) were added where indicated. Ca^{2+} (40 μM) was added to the experiments represented by the *lines c, d, e*. *Lines c* represent experiments conducted in the presence of the PT inhibitors 1 μM cyclosporin A, 200 μM ADP and 1 μg/ml oligomycin. *Lines a* represent control experiments in the absence of respiratory inhibitors and Ca^{2+}. Alamethicin (Alm.) (20 μg/ml) was added where indicated by the arrows.

Fig. 3 - PT induced by partial inhibition of respiratory chain complex II plus Ca^{2+} is inhibited by the antioxidant catalase. BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O to estimate $\Delta\Psi$. 40 μM MA (**Panel A**, *lines c, d*), 4 mM MMA (**Panel B**, *lines c, d*) or 300 μM 3-NP (**Panel C**, *lines c, d*) were added where indicated. Ca^{2+} (40 μM) was added to the experiments represented by *lines b, c* and *d*. *Lines c* represent experiments conducted in the presence of 1 μM catalase. *Lines a* represent control experiments in the absence of respiratory inhibitors and Ca^{2+}. FCCP (1 μM) was added where indicated by the arrows.

Fig. 4 - PT induced by partial inhibition of respiratory chain complex II plus Ca^{2+} is inhibited by NADH-linked substrates. In **Panel A**, BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O to estimate $\Delta\Psi$. 40 μM MA (*lines b-d*) and 40 μM Ca^{2+} (*lines a-d*) were added where indicated. *Line b* represents an experiment conducted in the presence of 5 μM rotenone and 10 mM pyruvate, 5 mM malate, 5 mM α-ketoglutarate and 5 mM glutamate (NADH-linked substrates). *Line c* represents an experiment conducted in the presence of NADH-linked substrates. FCCP (1 μM) was added where indicated by the arrow. In **Panel B**, BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium, and NAD(P)H fluorescence was measured as described in Materials and Methods. 40 μM MA (*lines a, b*) and 40 μM Ca^{2+} (*lines a, b*) were added where indicated by the arrows. 5 μM rotenone and NADH-linked substrates were added where indicated (*line a*). *Lines b* and *c* represent experiments in the presence of 1 μM cyclosporin A, 200 μM ADP and 1 μg/ml oligomycin. *Line c* represents a control experiment without the addition of Ca^{2+} or MA. Diamide (1 mM) was added to all experiments where indicated.

Fig. 5 - Partial inhibition of mitochondrial respiratory chain complexes I and III does not induce PT in the presence of Ca^{2+}. BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O to estimate $\Delta\Psi$. The experiments depicted in **Panels B** and **D** were conducted in the presence of the NADH-linked

substrates 10 mM pyruvate and 5 mM malate, instead of succinate. **Panels A** and **B**, mitochondrial oxygen consumption measurements in the presence of 1 mM ADP and varying concentrations of the complex III inhibitor antimycin A (**Panel A**) or the complex I inhibitor rotenone (**Panel B**). **Panels C** and **D**, effect of complex I and III inhibitors and Ca^{2+} on $\Delta\Psi$. 20 nM antimycin A (**Panel C**, *lines b, d, e*) or 5 nM rotenone (**Panel D**, *lines b, d, e*) were added where indicated. Ca^{2+} (40 μM) was added to the experiments represented in *lines c, d* and *e*. *Lines e* represent experiments conducted in the presence of the PT inhibitors 1 μM cyclosporin A, 200 μM ADP and 1 μg/ml oligomycin. *Lines a* represent control experiments in the absence of respiratory inhibitors and Ca^{2+}. FCCP (1 μM) was added where indicated by the arrows.

Fig. 6 - Isolated liver and kidney mitochondria are less sensitive to PT induced by partial respiratory chain complex II inhibition plus Ca^{2+}. Isolated rat liver (LM) (**Panels A** and **C**) or kidney (KM) (**Panels B** and **D**) mitochondria (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O. **Panels A** and **B**, mitochondrial oxygen consumption measurements in the presence of 1 mM ADP and varying concentrations of the complex II inhibitor MA. **Panels C** and **D**, effect of MA and Ca^{2+} on $\Delta\Psi$. MA (100 μM, *lines d*; 200 μM, *lines e* or 400 μM, *lines b* and *f*) was added where indicated. Ca^{2+} (100 μM) was added to the experiments represented by the *lines b-f*. *Lines b* represent experiments conducted in the presence of the PT inhibitors 1 μM cyclosporin A, 200 μM ADP and 1 μg/ml oligomycin. *Lines a* represent control experiments. FCCP (1 μM) was added where indicated by the arrows.

Fig. 7 - PT induced by partial inhibition of respiratory chain complex II plus Ca^{2+} is dependent on mitochondrial Ca^{2+} uptake. BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium containing 5 μM safranine O to estimate $\Delta\Psi$. MMA (4 mM) was added at 2 min (*lines d, e*) or at 7 min (*lines a-c*). Ca^{2+} (40 μM) was added at 2 min (*lines a-c*) or at 7 min (*lines d, e*). The experiments represented by the *lines a, c* and *e* were conducted in the presence of 10 mM NaCl. *Line a* represents an experiment in which 1 μM ruthenium red was added where indicated. FCCP (1 μM) was added where indicated by the arrow.

Fig. 8 - PT induced by partial inhibition of respiratory chain complex II plus Ca^{2+} is dependent on $\Delta\Psi$-driven Ca^{2+} uptake. BM (0.5 mg/ml) were incubated at 28°C in standard reaction medium in the presence of 100 nM FCCP and 2 μM Ca^{2+} ionophore A23187. 4 mM MMA (*lines b, d*) and 250 μM Ca^{2+} (*lines c, d*) were added where indicated. *Line a* represents a control experiment. A second addition of Ca^{2+} (500 μM) was made where indicated (*) (*lines c, d*). Alamethicin (Alm.) (20 μg/ ml) was added where indicated by the arrow (*line d*).

Fig. 9 - MMA-induced PC12 cell death is prevented by PT inhibitors. Cultured PC12 cells were incubated for 24 hours in the presence of 20 mM MMA, 1 μM CsA, 1 μM bongkrekic acid (BKA) and/or 0.1 μM FK506, as indicated. Total cell contents (**Panel A**, *empty bars*), non-viable cells (**Panel A**, *grey bars*) and medium pH (**Panel B**) were measured as described in Materials and Methods. * = $p < 0.05$, relative to control; # = $p < 0.05$ to MMA.

Fig. 10 - MMA-induced decrease in striatal slice viability is prevented by the PT inhibitor cyclosporin A. Rat striatal slices were incubated for 3 hours in the presence of 3 mM MMA, 1 μM CsA, and/or 1 μM antimycin A, as indicated. Slice viability was measured as described in Materials and Methods. * = $p < 0.01$, relative to control; # = $p < 0.01$ relative to MMA.

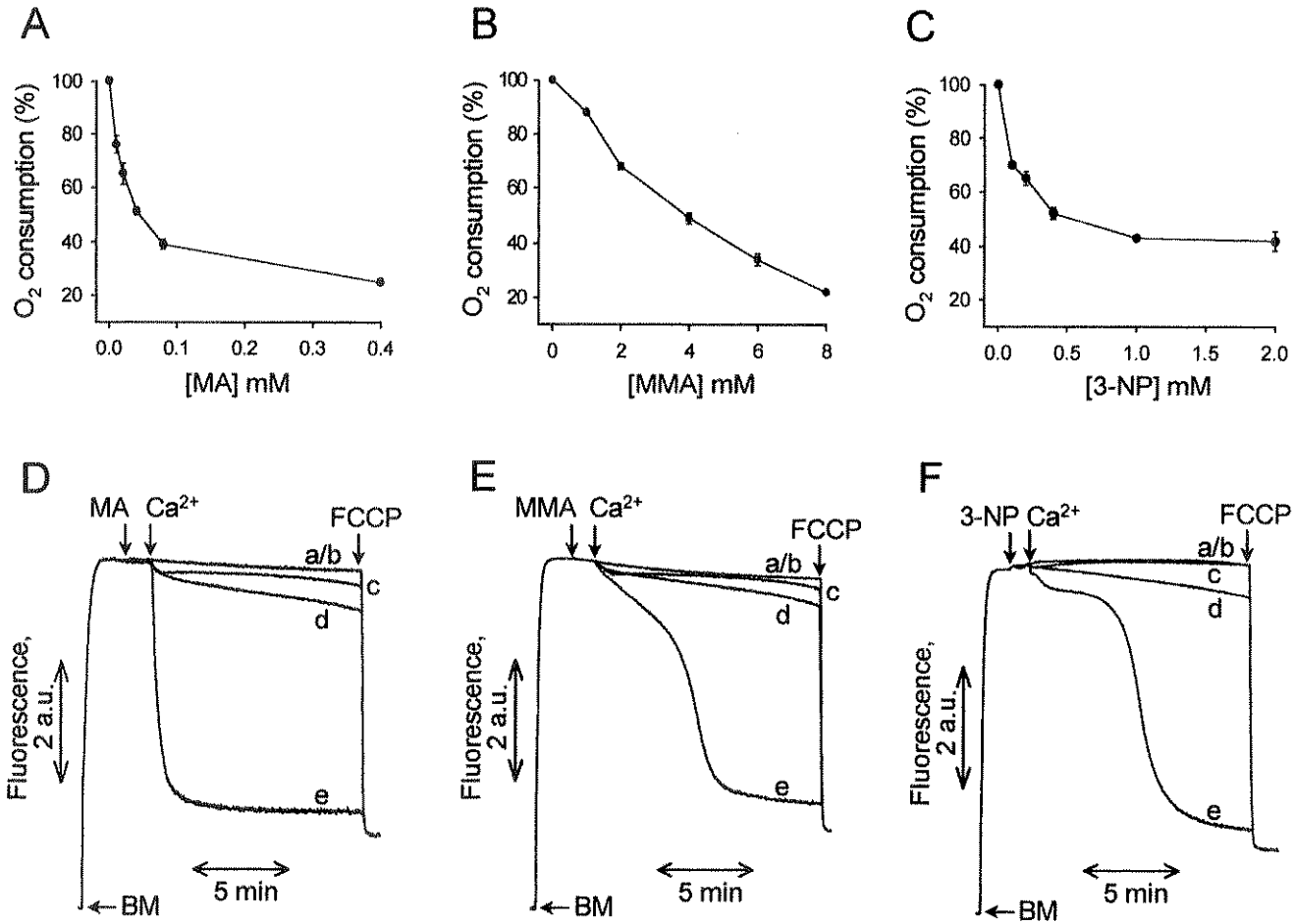


Fig. 1 - Maciel *et al.*, 2004.

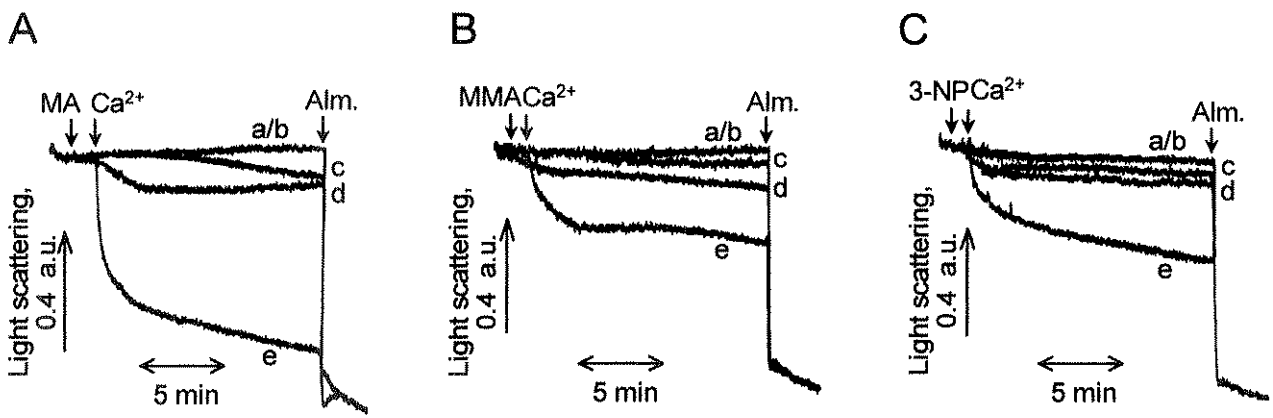


Fig. 2 - Maciel *et al.*, 2004.

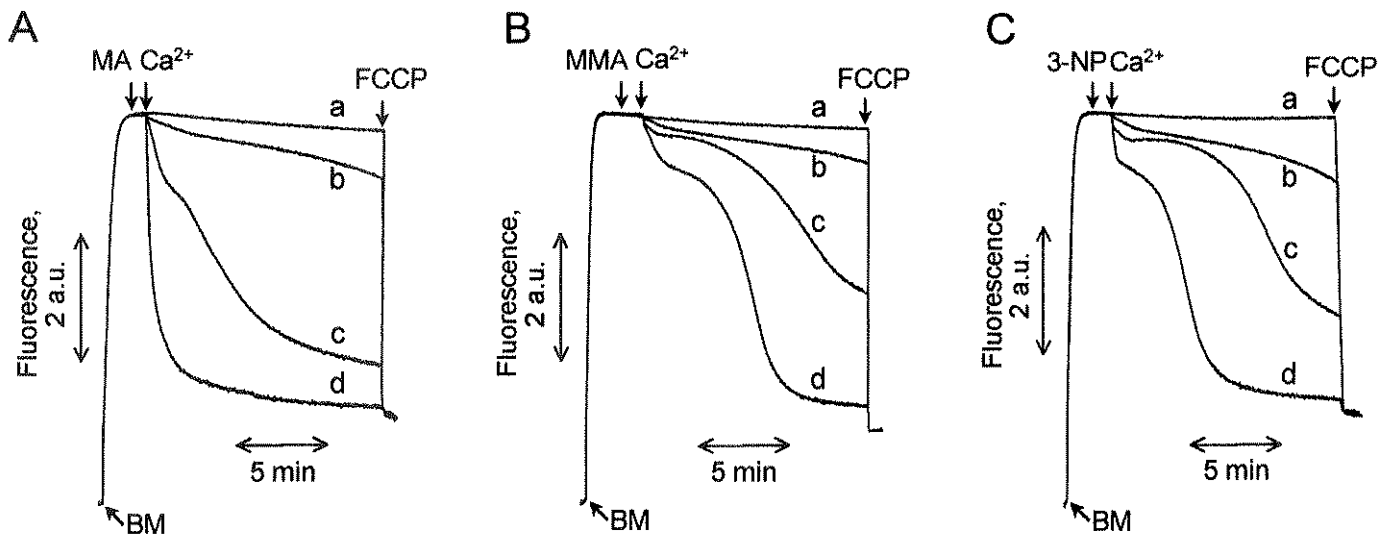


Fig. 3 - Maciel *et al.*, 2004.

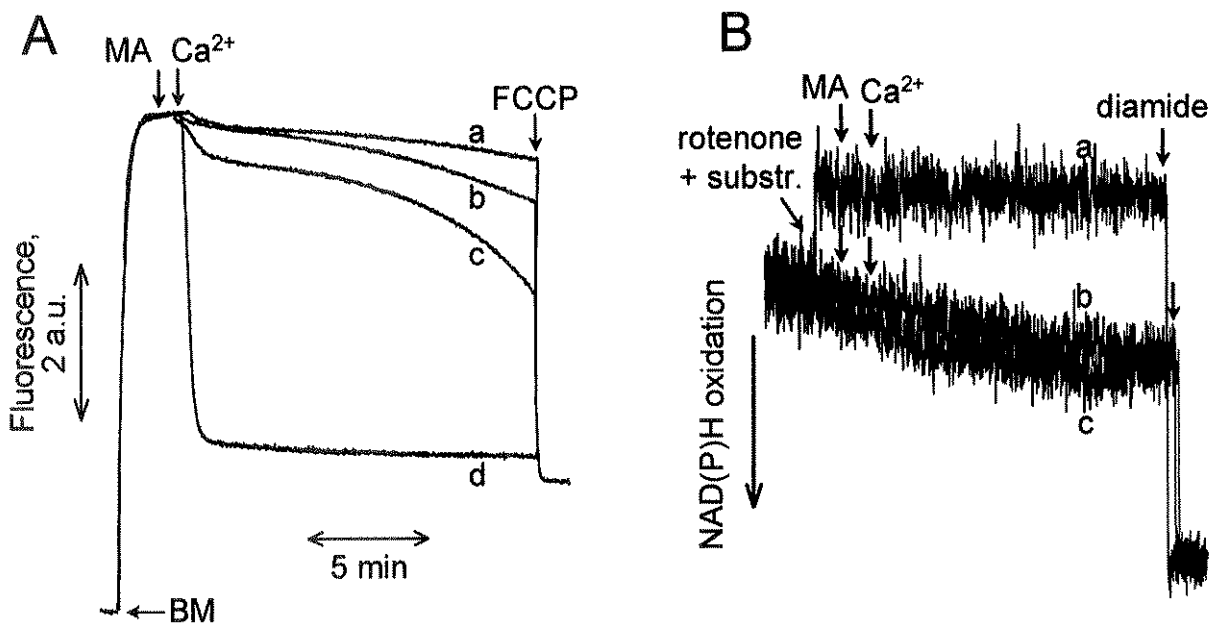


Fig. 4 - Maciel *et al.*, 2004.

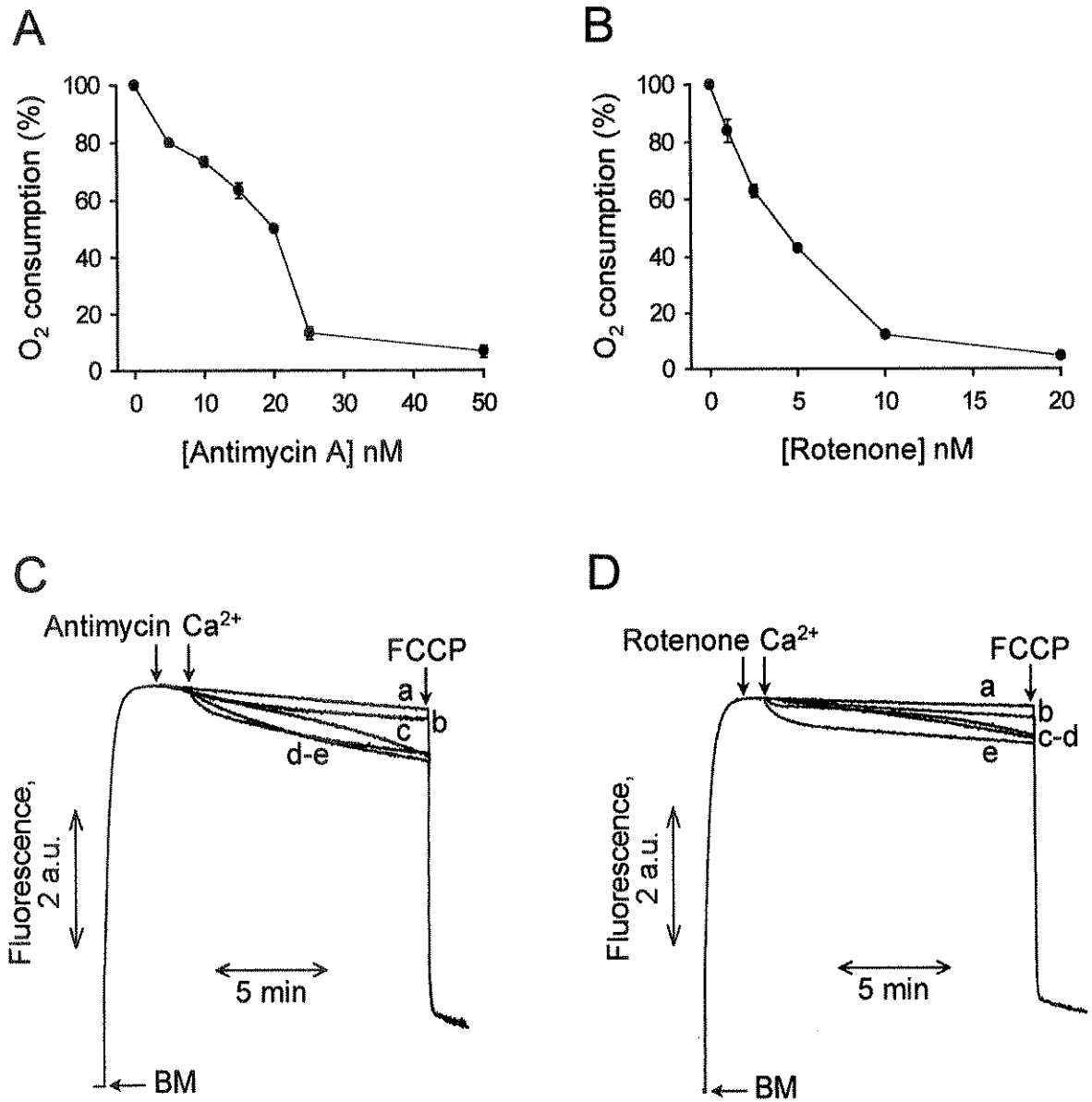


Fig. 5 - Maciel *et al.*, 2004.

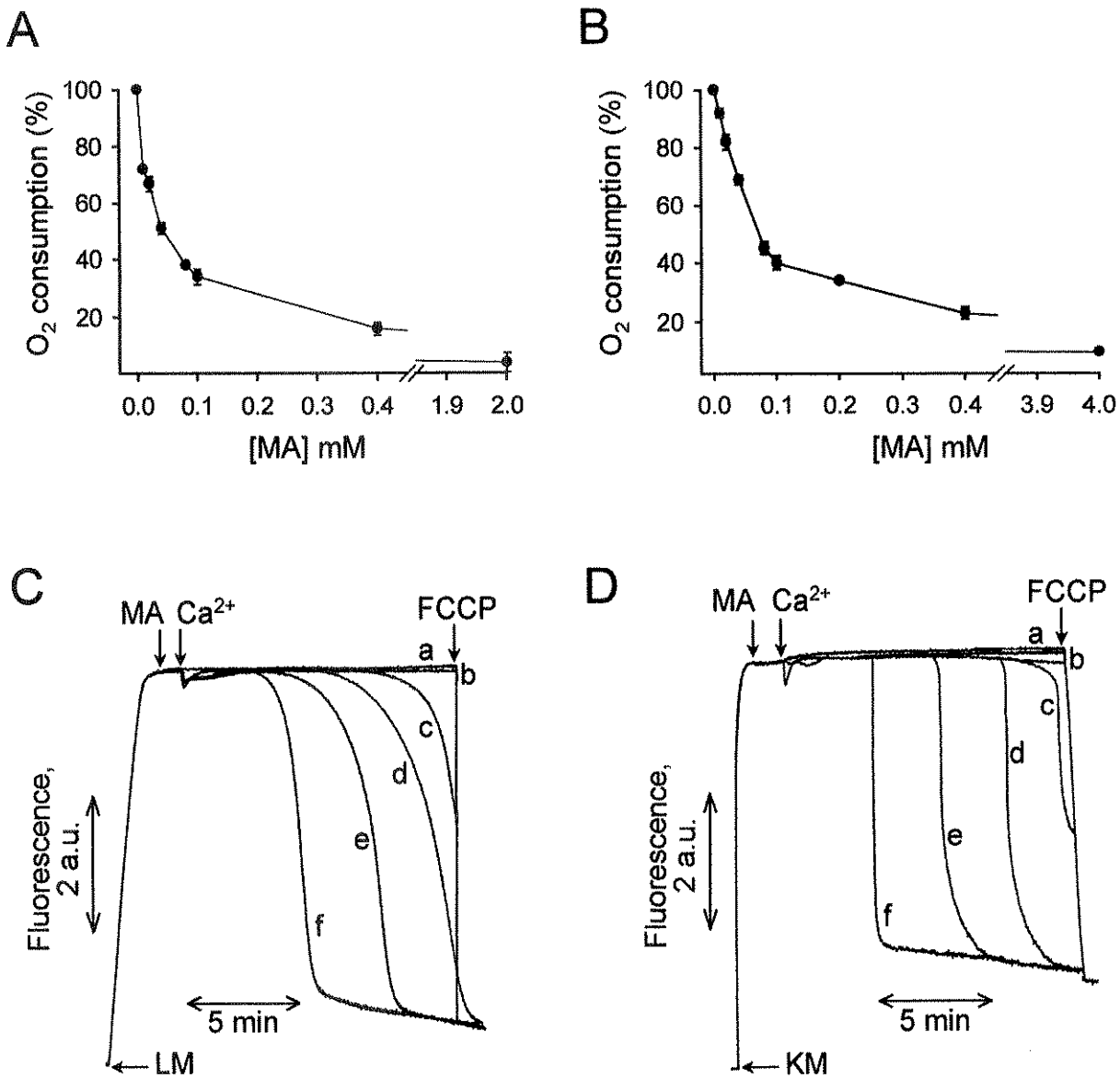


Fig. 6 - Maciel *et al.*, 2004.

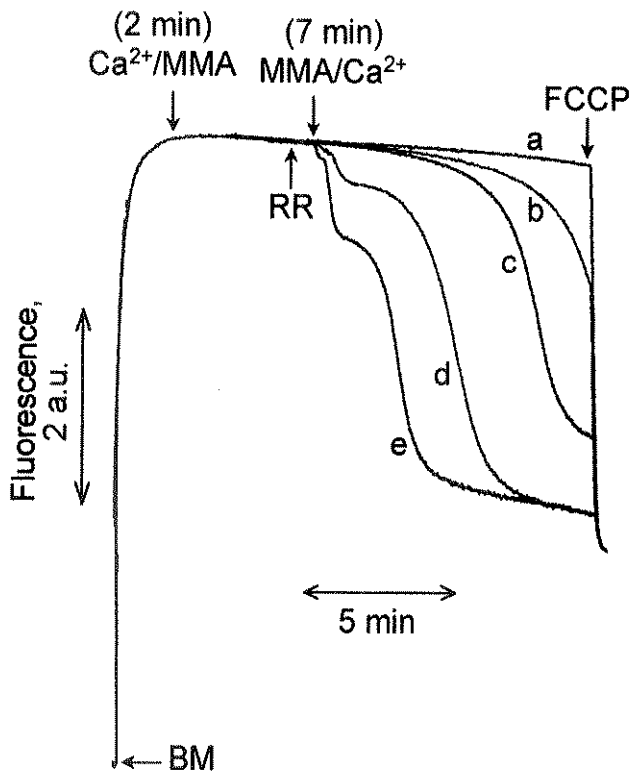


Fig. 7 - Maciel *et al.*, 2004.

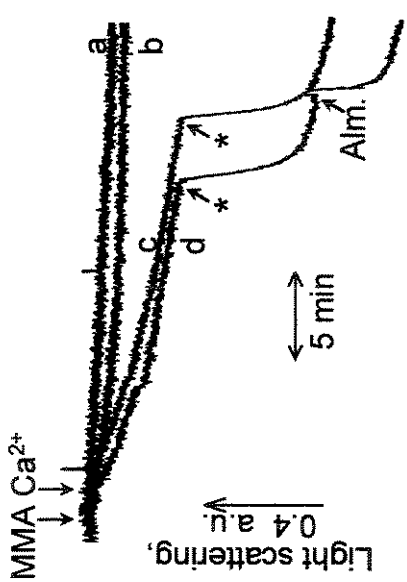


Fig. 8 - Maciel *et al.*, 2004.

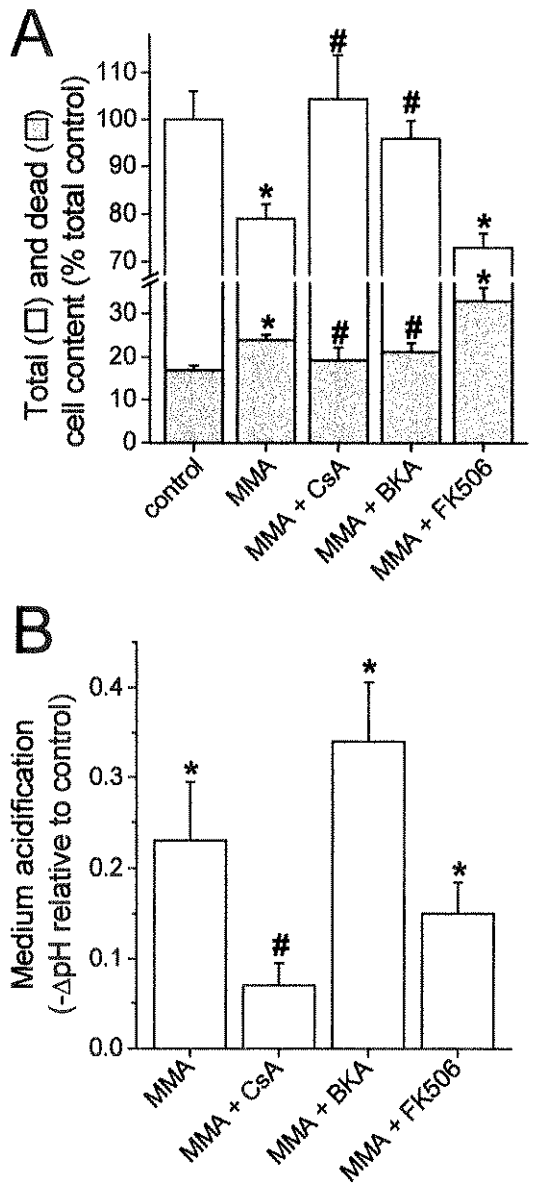


Fig. 9 - Maciel *et al.*, 2004.

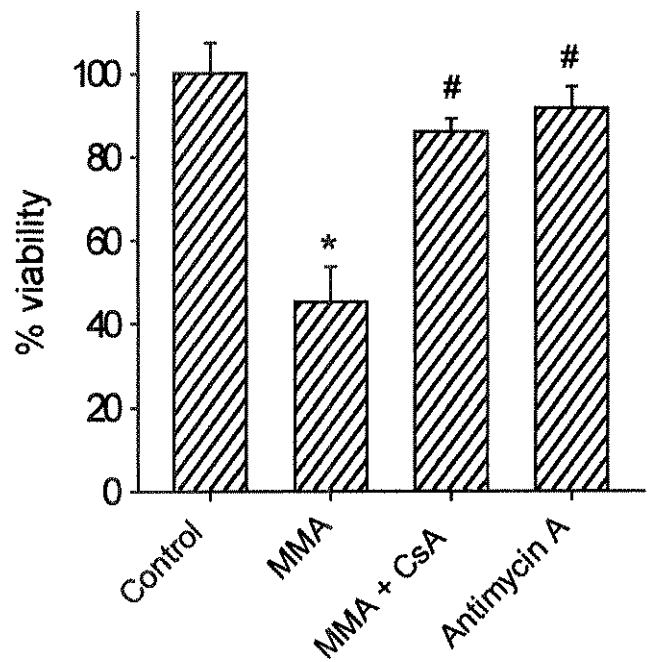


Fig. 10 - Maciel *et al.*, 2004.

4- DISCUSSÃO

A manutenção da homeostase de Ca^{2+} intracelular é crucial para a sobrevivência de neurônios e a disfunção mitocondrial pode estar envolvida em muitas desordens do sistema nervoso central (CHOI, 1995; FISKUM et al., 1999; NICHOLLS e BUDD, 2000). Durante o acúmulo de Ca^{2+} citosólico, a mitocôndria é a principal organela responsável pela captação de Ca^{2+} (NICHOLLS e BUDD, 2000). O aumento da concentração de Ca^{2+} na matriz mitocondrial pode induzir a PMT, que é caracterizada por uma permeabilização progressiva da membrana mitocondrial interna que, gradativamente, se torna permeável a prótons, suporte osmótico e até mesmo a pequenas proteínas, resultando em inchamento mitocondrial, rompimento da membrana externa e liberação de fatores apoptogênicos para o citosol (ZORATI e SZABÒ, 1995; GREEN e REED, 1998; KROEMER et al., 1998; KOWALTOWSKI et al., 2001).

Considerando evidências de que a PMT em mitocôndrias de fígado e coração pode ser mediada pela ação entre Ca^{2+} e EROs, levando à oxidação de grupos tiólicos de proteínas de membrana (VALLE et al., 1993; CASTILHO et al., 1995; GRIJALBA et al., 1999; KOWALTOWSKI et al., 2001) e o papel da mitocôndria como mediadora de processos oxidativos que ocorrem em algumas desordens neurológicas, examinamos a participação do estresse oxidativo na PMT induzida por Ca^{2+} em mitocôndrias isoladas de cérebro.

Os resultados presentes indicam a participação de estresse oxidativo na PMT induzida por Ca^{2+}, evidenciada por: i) inibição promovida pela catalase da dissipação do potencial elétrico de membrana ($\Delta\Psi$) induzida por Ca^{2+} e da liberação mitocondrial de Ca^{2+} (FIG. 2, ARTIGO 1); ii) aumento da produção de EROs após a PMT (FIG. 2, ARTIGO 1); e iii) depleção de nucleotídeos de piridina reduzidos e oxidação de lipídeos de membrana associada à PMT (FIGs. 5 e 6, ARTIGO 1).

O estresse oxidativo, detectado por meio da oxidação de diacetato de diclorodihidrofluoresceína (H_2DCF-DA), foi observado após a captação de Ca^{2+} e totalmente inibido pelos inibidores do poro de PMT, ADP e ciclosporina A (CsA) (FIG. 3, ARTIGO 1). Estes resultados sugerem que o Ca^{2+} estimula a produção de EROs em um sítio específico na membrana mitocondrial interna, onde EROs oxidariam grupos tióis de proteínas permitindo a abertura do poro de PMT (VALLE et al., 1993; CASTILHO et al.,

1995; KOWALTOWSKI et al., 1996; KOWALTOWSKI et al., 1998). Provavelmente estas EROs não sejam detectadas antes da PMT, pelas medidas de oxidação de H_2DCF-DA, devido aos seus efeitos local e instantâneo nas proteínas de membrana (STADTMAN, 1990).

Nossos resultados mostrando um aumento da produção de EROs mitocondrial seguido da despolarização da membrana contrastam com a esperada diminuição da produção de EROs após a despolarização da membrana mitocondrial em condições controle (BOVERIS e CHANCE, 1973; SKULACHEV, 1996). A despolarização na membrana aumenta o fluxo de elétrons na cadeia respiratória mitocondrial, diminuindo assim o estado reduzido dos carreadores de elétrons e a tensão de oxigênio. Isto deveria minimizar a produção de superóxido ao nível dos complexos I e III (BOVERIS e CHANCE, 1973; SKULACHEV, 1996).

O estresse oxidativo induzido pela PMT, observado em nossos estudos, provavelmente seja resultado do estímulo da produção de EROs mitocondrial e/ou uma deficiência nos sistemas antioxidantes mitocondriais. A PMT pode resultar em alterações estruturais da membrana mitocondrial interna, afetando a função da cadeia respiratória mitocondrial, incluindo o transporte de elétrons pela coenzima Q, e favorecendo a redução monoeletrônica do oxigênio (gerando radical superóxido) em passos intermediários da cadeia respiratória. Além disto, a PMT induz a liberação de citocromo *c* do espaço intermembrana, resultando em estímulo para a produção de superóxido (CAI e JONES, 1998). Sabe-se que a PMT, com despolarização da membrana mitocondrial, compromete o sistema antioxidante mitocondrial glutationa redutase/peroxidase e tioredoxina redutase/peroxidase, que depende do NADPH para reduzir H_2O_2 em água. A PMT resulta em dissipação do gradiente eletroquímico transmembrana de prótons e, nesta situação, a NADP transidrogenase não é capaz de manter o poder redutor mitocondrial (NADPH, glutationa e tioredoxina reduzidas), favorecendo o estresse oxidativo (VERCESI, 1987; HOECK e RYDSTROM, 1988). Além disto, a abertura do poro de PMT resulta em perda de NAD(P)H e de glutationa intramitocondrial para o meio extramitocondrial (IGBAVBOA et al., 1989). De fato, nossos estudos mostram uma diminuição, parcialmente devido à oxidação, na concentração de NAD(P)H após a PMT em mitocôndrias de cérebro de rato (FIG. 5, ARTIGO 1). Sugerimos que em mitocôndrias de cérebro, a PMT induzida pelo

estresse oxidativo, com conseqüente peroxidação lipídica de membrana (FIG. 6, ARTIGO 1), resulte em dano da fosforilação oxidativa mitocondrial e em permeabilização irreversível da membrana mitocondrial interna. Desta maneira, o estresse oxidativo e a disfunção mitocondrial induzidos pela PMT participariam da cascata de eventos que antecedem a morte neuronal em condições de acúmulo de Ca^{2+} citoplasmático (ORRENIUS et al., 2003).

A PMT induzida pelo Ca^{2+} foi estimulada por Na^{+} (FIG. 4, ARTIGO 1). Isto sugere que o aumento de Ca^{2+} livre citoplasmático acompanhado pelo aumento da concentração de Na^{+}, como observado em condições excitotóxicas (CHOI, 1988), pode levar a um estímulo da PMT e ao estresse oxidativo. Estes resultados estão de acordo com estudos prévios (DYKENS, 1994), mostrando que Na^{+} aumenta a produção de EROs em mitocôndrias isoladas de cérebro e cerebelo na presença de Ca^{2+}. Todavia, KRISTAL et al., 2000 não observaram efeito significativo de Na^{+} sobre a PMT em cérebro avaliada pelo inchamento mitocondrial. Esta observação aparentemente contrasta com nossos resultados, mostrando que o Na^{+} potencializa a dissipação do $\Delta\Psi$ induzido por Ca^{2+} e a liberação mitocondrial de Ca^{2+} (FIG. 4, ARTIGO 1). Provavelmente, as medidas do inchamento mitocondrial, secundário à entrada de suporte osmótico, podem não detectar pequenas alterações na permeabilidade da membrana mitocondrial (GUNTER e PFEIFFER, 1990), explicando os resultados de KRISTAL e colaboradores (2000).

A mitocôndria tem sido implicada na excitotoxicidade, incluindo o aumento da produção de EROs e participação na cascata de eventos que antecedem a morte celular por apoptose ou necrose (NICHOLLS e BUDD, 2000). Além disto, a PMT e excitotoxicidade têm sido implicadas na morte neuronal em modelos de isquemia, hipoglicemia e trauma cerebral (FRIBERG et al., 1998; UCHINO et al., 1998; FISKUM et al., 1999; MATSUMOTO et al., 1999). Com a finalidade de investigarmos o envolvimento da PMT na excitotoxicidade em ratos e camundongos, por meio da infusão estriatal de ácido quinolínico, um agonista endógeno de receptores NMDA, utilizamos ratos tratados sistemicamente com CsA (UCHINO et al., 1998) e camundongos que hiperexpressam Bcl-2, um inibidor de PMT (MARTINOU et al., 1994; MURPHY et al., 1996).

Em nossos estudos, não observamos nenhum efeito protetor de Bcl-2 (FIGURA 1, ARTIGO 2) ou CsA (FIGURA 2, ARTIGO 2) nas lesões estriatais promovidas por ácido quinolínico, indicando que a excitotoxicidade não é dependente da PMT neste modelo experimental de excitotoxicidade. Os resultados encontrados neste trabalho estão de acordo com resultados anteriores, obtidos em cultura de células granulosas cerebelares, mostrando que CsA e bongkrekato (BKA), um inibidor do poro de PMT por ligar-se ao translocador de nucleotídeo de adenina, um suposto componente do poro de PMT (MARCHETTI et al., 1996; BUDD et al., 2000; HAWORTH e HUNTER, 2000), não inibem a lesão celular induzida por glutamato (CASTILHO et al., 1998). De acordo com nossos resultados, um efeito limitado de CsA na morte celular induzida por glutamato em cultura de neurônios do hipocampo foi recentemente observado por BRUSTOVETSKY e DUBINSKY, 2000. Provavelmente a falta de efeito da CsA na lesão estriatal induzida por ácido quinolínico em ratos não seja devido à falha de inibição da PMT *in vivo*, já que mitocôndrias isoladas de cérebro de ratos tratados com CsA apresentam resistência à PMT induzida por Ca^{2+} quando comparadas com organelas isoladas de ratos controles (FIG. 3A, ARTIGO 2).

A hiperexpressão de Bcl-2 em linhagens celulares resulta em proteção contra a PMT induzida por Ca^{2+} (MURPHY et al., 1996; SHIMIZU et al., 1998; ZAMZAMI et al., 1998; KOWALTOWSKI et al., 2000) e resulta também em proteção contra à apoptose, a qual envolve a liberação de fatores apoptogênicos mitocondriais como o citocromo *c* (MARTINOU e GREEN, 2001). Vários estudos têm demonstrado que camundongos transgênicos que hiperexpressam Bcl-2 apresentam proteção contra a morte celular promovida por estresse oxidativo (MERAD-SAIDOUNE et al., 1999), inibição da cadeia respiratória (YANG et al., 1998; BOGDANOV et al., 1999) e privação de glicose (SCHIERLE et al., 1999). Entretanto, YANG et al., 2000 não observaram inibição da PMT em mitocôndrias isoladas de fígado de camundongos transgênicos que hiperexpressam Bcl-2 sob o controle da enolase específica de fígado. Nossos resultados com camundongos transgênicos que hiperexpressam Bcl-2 sugerem que a morte estriatal induzida por ácido quinolínico não é dependente da rota apoptótica de morte celular sensível a Bcl-2.

Em nossos experimentos, escolhemos injetar uma concentração e volume de ácido quinolínico que causasse lesão em aproximadamente 50% do volume estriatal, já que esperávamos observar um efeito protetor da CsA e Bcl-2 em neurônios estriatais. A lesão (morte celular) é completa no local onde foi inserida a agulha da infusão e, ao redor da infusão por ácido quinolínico, há uma penumbra onde poucos neurônios sobreviveram, local este onde esperávamos observar os efeitos protetores de CsA e Bcl-2 (NAKAO et al., 1996; DIRNAGI et al., 1999). Assim sendo, se a CsA e/ou Bcl-2 tivessem manifestado qualquer efeito neuroprotetor em baixas concentrações de ácido quinolínico, seria esperado que estes agentes inibissem a morte neuronal na zona de penumbra.

Os resultados encontrados no ARTIGO 2, indicando que a PMT não está envolvida na morte celular induzida por ácido quinolínico, são inesperados, visto que o hiperestímulo de receptores NMDA resulta em um aumento do influxo de Ca^{2+} e Na^{+} seguido do acúmulo de Ca^{2+} mitocondrial (NICHOLLS e BUDD, 2000) e aumento da produção de EROs (CASTILHO et al., 1999), situações que favorecem a PMT (ZORATTI e SZABÒ, 1995; KOWALTOWSKI et al., 2001; ARTIGO 1). Todavia, uma possível explicação para a não participação da PMT em nossos experimentos é a presença de inibidores endógenos neste fenômeno *in vivo*, como os nucleotídeos de adenina. De fato, observamos inibição da PMT induzida por Ca^{2+} *in vitro* na presença de concentrações micromolares de ATP e, em menor extensão, de ADP (FIG. 3B, ARTIGO 2). Outra possibilidade é que, sob nossas condições experimentais, as vias mitocondriais de morte neuronal, incluindo PMT, são desviadas por vias intracelulares que eventualmente resultam em morte celular. Nossos resultados sugerem que em situações em que ocorre depleção intracelular de ATP e ADP, como na isquemia cerebral (UCHINO et al., 1998; MATSUMOTO et al., 1999) e hipoglicemia (FRIBERG et al., 1998), pode ocorrer a participação da PMT na excitotoxicidade.

Considerando que a inibição do complexo II da cadeia transportadora de elétrons está envolvida na patogênese de algumas doenças neurológicas (BEAL, 1998), como na doença de Huntington (GU et al., 1996; BROWNE et al., 1997) e na acidemia metilmalônica, investigamos os efeitos da inibição do complexo II da cadeia respiratória mitocondrial e sua influência na PMT no SNC. A acidemia metilmalônica é uma desordem

metabólica que envolve déficit na conversão de metilmalonil-CoA em succinil-CoA (MATSUI et al., 1983; LEDLEY, 1990; SWEETMAN e WILLIAMS, 2001), resultando em manifestações neurológicas como encefalopatias e coma nos primeiros meses de vida.

A fim de monitorarmos os efeitos de inibidores do complexo II em mitocôndrias de cérebro, utilizamos malonato (MA), um inibidor competitivo, 3-NP, um inibidor não competitivo e irreversível da respiração mitocondrial, e metilmalonato (MMA), que inibe a respiração mitocondrial através da produção de metabólitos intramitocondriais (OKUM et al., 2002). Os resultados encontrados mostram que 50% da inibição da respiração mitocondrial por MA, MMA ou 3-NP têm um efeito sinérgico com a adição de baixas concentrações de Ca^{2+}, levando à perda do $\Delta\Psi$ (FIG. 1, ARTIGO 3). Esta perda do $\Delta\Psi$ é causada pela PMT, pois é acompanhada pelo inchamento da mitocôndria e pôde ser prevenida por CsA e ADP (FIG. 2, ARTIGO 3). Interessantemente, mitocôndrias de fígado e rim requerem uma maior inibição da cadeia respiratória mitocondrial por MA, além de maiores concentrações de Ca^{2+} (FIG. 6, ARTIGO 3) para indução da PMT, sugerindo uma maior sensibilidade do cérebro à indução da PMT por inibidores do complexo II. Estas evidências contribuem para trabalhos que descrevem diferentes características da PMT e captação mitocondrial de Ca^{2+} no cérebro (SIESJÖ e SIESJÖ, 1996; ANDREYEV e FISKUM, 1999; BRUSTOVETSKY et al., 2003).

Em mitocôndrias de cérebro, a perda do $\Delta\Psi$ e o inchamento mitocondrial promovidos por Ca^{2+} e inibidores do complexo II são parcialmente prevenidos pelo antioxidante catalase (FIG. 3, ARTIGO 3), indicando que a PMT está associada com a produção de EROs, como observado em vários modelos (KOWALTOWSKI et al., 1996; CASTILHO et al., 1998; ARTIGO 1). Além disto, a PMT promovida por MMA e Ca^{2+} é prevenida por substratos dependentes de NADH, mesmo na presença de rotenona, um inibidor do complexo I. Baseados nestes resultados, acreditamos que a PMT promovida por MMA é prevenida pelo acúmulo de NAD(P)H intramitocondrial, aumentando sua capacidade redox (CHALMERS e NICHOLLS, 2003). Os resultados apresentados estão de acordo com resultados de ZAGO et al., 2000, que demonstram que a PMT pode ser inibida pelo acúmulo intramitocondrial de NADH e NADPH.

As EROs na mitocôndria são aumentadas na presença de inibidores da respiração mitocondrial e Ca^{2+}, tendo como exemplo o aumento de EROs pela inibição do complexo I por rotenona na presença de concentrações micromolares de Ca^{2+} (STARKOV et al., 2002; SOUSA et al., 2003). Entretanto, este estresse oxidativo não é necessariamente o causador da PMT em nossas condições (ARTIGO 3), como indicado pela ausência de permeabilização mitocondrial, quando Ca^{2+} foi adicionado na presença de rotenona ou antimicina A (FIG. 5, ARTIGO 3). Assim, somente a inibição da respiração e/ou o estresse oxidativo não são suficientes para causar a PMT em nosso modelo experimental. A causa para o aumento de suscetibilidade para a PMT parece envolver a perda de habilidade mitocondrial para responder adequadamente com estímulo respiratório diante da captação de Ca^{2+}, quando a respiração mantida por succinato encontra-se parcialmente inibida. De fato, o estímulo do ciclo de Ca^{2+} através do efluxo pelo trocador Ca^{2+}/Na^{+} intensifica o efeito de MMA na indução da PMT (FIG. 7, ARTIGO 3). A PMT também é menor quando a mitocôndria acumula Ca^{2+} antes do MMA ser adicionado (FIG. 7, ARTIGO 3) e a PMT induzida por MMA não é observada em mitocôndrias desenergizadas de cérebro, em que a entrada de Ca^{2+} foi promovida pelo A23187, um ionóforo de Ca^{2+} (FIG. 8, ARTIGO 3). Assim, sugerimos que, nas condições em que a respiração mitocondrial está parcialmente inibida por MMA, o aumento no transporte de elétrons devido à captação de Ca^{2+} leva ao estresse oxidativo e à PMT. Estes resultados sugerem que a diminuição do transporte de elétrons pela succinato desidrogenase (SDH) leva a mitocôndria a uma maior sensibilidade a oxidantes endógenos e à captação de Ca^{2+} mediada pelo $\Delta\Psi$.

Utilizando um modelo de acidemia metilmalônica em células PC12 intactas, confirmamos a ocorrência de PMT por meio da prevenção, por CsA, da morte celular induzida por MMA (FIG. 9, ARTIGO 3). A CsA também previne a morte celular induzida por MMA em fatias estriatais (FIG. 10, ARTIGO 3). Além disto, BKA também previne a morte celular induzida por MMA. Considerando que o BKA, que inibe a fosforilação oxidativa por inibir o translocador de nucleotídeo de adenina, promove a conservação da viabilidade celular na presença de MMA, conclui-se que o efeito citotóxico de MMA não é devido primariamente à deficiência do metabolismo energético destas células. De fato, o meio de incubação na presença de BKA é significantemente acidificado comparado com os controles (FIG. 9, ARTIGO 3), sugerindo que estas células estão ativamente mais

glicolíticas e gerando mais lactato. Além disto, a antimicina A, um inibidor do complexo III da cadeia respiratória mitocondrial, não se mostrou eficaz na indução da PMT e diminuição da viabilidade de fatias estriatais (FIG. 10, ARTIGO 3). Assim sendo, a morte celular neste modelo parece ser causada pela PMT promovida pela presença de MMA, e não diretamente devido aos efeitos metabólicos do MMA.

Em nossos estudos, por meio de modelos experimentais *in vitro* e *in vivo* associados a mudanças no metabolismo energético, incluindo isquemia cerebral, acidemia metilmalônica e doença de Huntington, propomos que a disfunção mitocondrial e a PMT induzida pelo estresse oxidativo participam da liberação de sinais apoptogênicos que determinam a morte celular associada ao acúmulo de Ca^{2+} citosólico. Além disto, sugerimos o envolvimento da integridade mitocondrial no modelo de morte neuronal da acidemia metilmalônica; especulando que inibidores da PMT são potencialmente candidatos a intervenções terapêuticas para prevenção de danos neuronais em pacientes com acidemia metilmalônica e, possivelmente, na doença de Huntington.

5- REFERÊNCIAS BIBLIOGRÁFICAS

ADAMS, J.M.; CORY, S. The Bcl-2 protein family: arbiters of cell survival. **Science**, 281: 1322-6, 1998.

ALBIN, R.L.; GREENAMYRE, J.T. Alternative excitotoxic hypotheses. **Neurology**, 42: 733-8, 1992.

ANDREYEV, A.; FISKUM, G. Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain versus liver. **Cell Death Differ**, 6: 825-32, 1999.

ANGLADE, P.; VYAS, S.; JAVOY-AGID, F.; HERRERO, M.T.; MICHEL, P.P.; MARQUEZ, J.; MOUATT-PRIGENT, A.; RUBERG, M.; HIRSH, E.C.; AGID, Y. Apoptosis and autophagy in nigral neurons of patients with Parkison's disease. **Histol Histopathol**, 12: 25-31, 1997.

ANTONSSON, B.; CONTI, F.; CIAVATTA, A.; MONTESSUIT, S.; LEWIS, S.; MARTINOU, I.; BERNASCOVI, L.; BERNARD, A.; MERMOD, J.J.; MAZZEI, G.; MAUNDRELL, K.; GAMBALE, F.; SADOUL, R.; MARTINOU, J.C. Inhibition of Bax channel-forming activity by Bcl-2. **Science**, 277: 370-2, 1997.

AUER, R.N. Insulin, blood glucose levels, and ischemic brain damage. **Neurology**, 51: 39-43, 1998.

BEAL, M.F. Mitochondrial dysfunction in neurodegenerative diseases. **Biochim Biophys Acta**, 1366: 211-23, 1998.

BEAL, M.F.; KOWALL, N.W.; ELLISON, D.W.; MAZUREK, M.F.; SWARTZ, K.J.; MARTIN, J.B. Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. **Nature**, 321: 168-71, 1986.

BEAL, M.F.; KOWALL, N.W.; SWARTZ, K.J.; FERRANTE, R.J.; MARTIN, J.B. Systemic approaches to modifying quinolinic acid striatal lesions in rats. **J Neurosci**, 8: 3901-8, 1988.

BERRIDGE, M.J. Inositol triphosphate and calcium signalling. **Nature**, 361: 315-25, 1993.

BOGDANOV, M.B.; FERRANTE, R.J.; MUELLER, G.; RAMOS, L.E.; MARTINOU, J.C.; BEAL, M.F. Oxidative stress is attenuated in mice overexpressing Bcl-2. **Neurosci Lett**, 262: 33-6, 1999.

BOVERIS, A.; CADENAS, E.; STOPPANI, A.O. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. **Biochem J**, 156: 434-44, 1976.

BOVERIS, A.; CHANCE, B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. **Biochem J**, 134: 707-16, 1973.

BROWNE, S.E.; BOWLING, A.C.; MACGARVEY, U.; BAIK, M.J.; BERGER, S.C.; MUQIT, M.M.; BIRD, E.D.; BEAL, M.F. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. **Ann Neurol**, 41: 646-53, 1997.

BRUSTOVETSKY, N.; BRUSTOVETSKY, T.; PURL, K.J.; CAPANO, M.; CROMPTON, M.; DUBINSKY, J.M. Increased susceptibility of striatal mitochondria to calcium-induced permeability transition. **J Neurosci**, 23: 4858-67, 2003.

BRUSTOVETSKY, N.; DUBINSKY, J.M. Limitations of cyclosporin A inibition of the permeability transition in CNS mitochondria. **J Neurosci**, 20: 8229-37, 2000.

BUDD, S.L.; TENNETI, L.; LISHNAK, T.; LIPTON S.A. Mitochondrial and extramitochondrial apoptotic signalling pathways in cerebrocortical neurons. **Proc Natl Acad Sci USA**, 97: 6161-6, 2000.

BURSCH, W.; ELLINGER, A.; GERNER, C.; FROHWEIN, U.; SCHULTE-HERMANN, R. Programated cell death (PCD). Apoptosis, autophagic PCD, or others? **Ann N Y Acad Sci**, 926: 1-12, 2000.

CAI, J.; JONES, D.P. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome *c* loss. **J Biol Chem**, 273: 11401-4, 1998.

CARAFOLI, E. Intracellular calcium homeostasis. **Annu Rev Biochem**, 56: 395-433, 1987.

CASTILHO, R.F.; KOWALTOWSKI, A.J.; MEINICKE, A.R.; BECHARA, E.J.H.; VERCESI, A.E. Permeabilization of inner mitochondrial membrane by Ca^{2+} ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria. **Free Radical Biol Med**, 18: 479-86, 1995.

CASTILHO, R.F.; KOWALTOWSKI, A.J.; VERCESI, A.E. The irreversibility of inner mitochondrial membrane permeabilization by Ca^{2+} plus prooxidants is determined by the extend of membrane protein thiol cross-linking. **J Bionerg Biomembr**, 28: 523-9, 1996.

CASTILHO, R.F.; HANSSON, O.; WARD, M.W.; BUDD, S.L.; NICHOLLS, D.G. Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. **J Neurosci**, 18: 10277-86, 1998.

CASTILHO, R.F.; WARD, M.W.; NICHOLLS, D.G. Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells. **J Neurochem**, 72: 1394-401, 1999.

CATALDO, A.M.; BARNETT, J.L.; BERMAN, S.A.; LI, J.; QUARLESS, S.; BURSZTAJN, S.; LIPPA, C.; NIXON, R.A. Gene expression and cellular contend of cathepsin D in Alzheimer's disease brain: evidence for early upregulattion of endosomal-lysosomal system. **Neuron**, 14: 671-80, 1995.

CHALMERS, R.A.; LAWSON, A.M. Organic acids in man. In: **Analitical chemistry, biochemistry and diagnosis of the organic acidurias**. London: Chapmam and Hall, 1982. p. 221-9.

CHALMERS, S.; NICHOLLS, D.G. The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. **J Biol Chem**, 278: 19062-70, 2003.

CHOI, D.W. Calcium mediated neurotoxicity: relantionship to specific channel types in ischemic damage. **Trends in Neurosci**, 11: 465-9, 1988.

CHOI, D.W. Calcium: still center-stage in hypoxic-ischemic neuronal death. **Trends Neurosci**, 18: 58-60, 1995.

CLAPHAM, D.E. Intracellular calcium. Replenishing the stores. **Nature**, 375: 634-5, 1995.

CONNERN, C.P.; HALESTRAP, A.P. Recruitment of mitochondrial cyclophylin to the mitochondrial inner membrane under conditions of oxidative stress that that enhance the opening of a calcium-sensitive non-specific channel. **Biochem J**, 302: 321-24, 1994.

COYLE, J.T.; PUTTFARCKEN, P. Oxidative stress, glutamate, and neurodegenerative disorders. **Science**, 262: 689-95, 1993.

CROMPTON, M.; ELLINGER, H.; COSTI, A. Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. **Biochem J**, 255: 357-60, 1988.

DAWSON, T.L.; GORES, G.J.; NIEMINEN, A.L.; HERMAN, B.; LEMASTERS, J.J. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. **Am J Physiol**, 264: 961-7, 1993.

DE MEIS, L.; VIANNA, A.L. Energy interconversion by the Ca^{2+} -dependent ATPase of the sarcoplasmic reticulum. **Annu Rev Biochem**, 28: 275-92, 1979.

DIRNAGI, U.; IADECOLA, C.; MOSKOWITZ, M.A. Pathobiology of ischaemic stroke: an integrated view. **TINS**, 22: 391-7, 1999.

DINGLEDINE, R.; MCBAIN, C.J. Three Classes of Ionotropic Glutamate Receptor. In: **Basic Neurochemistry**: Philadelphia: Lippincott-Raven, 1999. p. 304-43.

DIXON, K.C. Ischaemia and the neuron. **Neurohistochemistry**. C. W. M. Adams. Amsterdan, Elsevier, 558-98, 1965.

DU, C.; FANG, M.; LI, Y.; LI, L.; WANG, X. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. **Cell**, 102: 33-42, 2000.

DUGAN, L.L.; CHOI, D.W. Hypoxic-ischemic brain injury and oxidative stress. In: **Basic Neurochemistry**: Philadelphia: Lippincott-Raven, 1999. p. 680-700.

DUNN, W.A.Jr. Autophagy and related mechanism of lysossome-mediated protein degradation. **Trends Cell Biol**, 4: 139-43, 1994.

DURAN, M.; DORLAND, L.; BREE, P.K.; BERGER, R. Selective screening for aminoacid disorders. **Eur J Pediatr** 153: 33-7, 1994.

DYKENS, J.A. Isolated cerebral and cerebral mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: Implications for neurodegeneration. **J Neurochem**, 63: 584-91, 1994.

ESFANDIARI, A.; SOIFIYOUDINE, D.; PATURNEAU-JOUAS, M. Inhibition of fatty acid beta-oxidation in rat brain cultured astrocytes exposed to the neurotoxin 3-nitropropionic acid. **Dev Neurosci**, 19: 312-20, 1997.

EVANGELIOU, A.; STUMF, D.A.; PARKS, J.K. Citrate syntase inhibition by acyl CoA esters. **Ann Neurol**, 18: 383-4, 1985.

FAROOQUI, A. A.; HAUND, S. E.; HORROCKS, L. A. Ischemia and Hipoxia. In: SIEGEL, G.S.; AGRANOFF, B.W.; ALBERS, R.W.; MOLLINOFF, P.B. **Basic Neurochemistry**. New York: Raven Press, 1995. p. 867-83.

FENTON, W.A.; GRAVEL, R.A.A.; ROSENBLATT, D.S. Disorders of propionate and methylmalonate metabolism. In: SCRIVER, C.R.; BEAUDET, A.; SKY, W.S.; VALLE, D. **The Metabolic and Molecular Bases of Inherited Disease**. New York: McGraw-Hill, 2001. p. 2165-93.

FISKUM, G.; MURPHY, A.N.; BEAL, M.F. Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. **J Cereb Blood Flow Metab**, 19: 351-69, 1999.

FRIBERG, H.; FERRAND-DRAKE, M.; BENGTSSON, F.; HALESTRAP, A.D.; WIELOCH, T. Cyclosporin A, but not FK-506, protects mitochondria and neurons against hypoglicemia damage and implicates the mitochondrial permeability transition. **J Neurosci**, 18: 5151-59, 1998.

GINSBERG, M. D.; BUSTO, R. Rodent models of cerebral ischemia. **Stroke**, 20: 1627-42, 1989.

GINSBERG, M.D. Animal models of global and focal cerebral ischemia. In: WELCH, K.M.A.; REIS, D.J.; CAPLAN, L.R.; SIESJÖ, B.K.; WEIR, B. **Primer on cerebrovascular diseases**. San Diego: Academic Press, 1997.

GHOLSON, R.K.; UEDA, I.; OGASAWARA, H.; HENDERSON, L.M. The enzymatic conversion of quinolinic acid to nicotinic acid mononucleotide in mammalian liver. **J Biol Chem**, 239: 1208-14, 1964.

GLASGOW, A.M.; CHASE, P. Effect of propionic acid on fatty acid oxidation and ureagenesis. **Pediatr Res**, 19: 633-86, 1976.

GREEN, D.R.; REED, J.C. Mitochondria and apoptosis. **Science**, 281: 1309-12, 1998.

GREGERSEN, N. The specific inhibition of the pyruvate dehydrogenase complex from pig kidney by propionyl- CoA and isovaleryl- CoA. **Biochem Med**, 26: 20-7, 1981.

GRIFFITHS, E.J.; HALESTRAP, A.P. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. **Biochem J** , 307: 93-8, 1995.

GRIJALBA, M.T.; VERCESI, A.E.; SCHEREIER, S. Ca^{2+}-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca^{2+}-stimulated generation of reactive oxygen species by the respiratory chain. **Biochemistry**, 38: 13279-87, 1999.

GU, M.; GASH, M.T.; MANN, V.M.; JAVOY-AGID, F.; COOPER, J.M.; SCHAPIRA, A.H. Mitochondrial defect in Huntington's disease caudate nucleus. **Ann Neurol**, 39: 385-9, 1996.

GUNTER, K.K.; GUNTER, T.E. Transport of calcium by mitochondria. **J Bioenerg Biomembr**, 26: 471-85, 1994.

GUNTER, T.E.; PFEIFFER, D.R. Mechanisms by which mitochondria transport calcium. **Am J Physiol**, 258: C755-86, 1990.

GUTTERIDGE, J.M.; HALLIWELL, B. Free radicals and antioxidants in the year 2000. A historical look to the future. **Ann N Y Acad Sci**, 899: 136-47, 2000.

HACKEL, D.B.; JENNINGS, R.B. The heart. In: RUBIN, F.; FABER, D.J. **Pathology**. Philadelphia: Lippincot, 1988. p. 515-25.

HATEFI, Y. The mitochondrial electron transport and oxidative phosphorylation system. **Annu Rev Biochem**, 54: 1015-69, 1985.

HAWORTH, R.A.; HUNTER, D.R. Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria. **J Bioenerg Biomembr**, 32: 91-6, 2000.

HOECK, J.B.; RYDSTROM, J. Physiological roles of nicotinamide nucleotide transhydrogenase. **Biochem J**, 254:1-10, 1988.

HOFFMANN, Gc.F.; MEIER-AUGENSTEIN, W.; STOCKER, S.; SURTEES, R.; RATTING, D.; NYHAN, W.L. Physiology and pathophysiology of organic acids in cerebrospinal fluid. **J Inher Metab Dis**, 16: 648-69, 1993.

HOFFMANN, G.F. Selective screening for inborn errors of metabolism: past, present and future. **Eur J Pediatr**, 153: 2-8, 1994.

HOLLMANN, M.; HEINEMANN, S. Cloned glutamate receptors. **Annu Rev Neurosci**, 17: 31-108, 1994.

HUNTER, A.J.; GREEN, A.R.; CROSS, A.J. Animal models of acute ischaemic stroke: can they predict clinically successful neuroprotective drugs? **Trends Pharmacol Sci**, 16: 123-8, 1995.

HUNTER, D.R.; HAWORTH, R.A. The Ca^{2+}-induced membrane transition in mitochondria: The protective mechanisms. **Arch Biochem Biophys**, 195: 453-59, 1979.

IGBAVBOA, U.; ZWIZINSKI, C.; PFEIFFER, D.R. Release of mitochondrial matrix proteins through a Ca^{2+}-requiring cyclosporin sensitive pathway. **Biochem Biphys Res Commun**, 161: 619-25, 1989.

de JONG, A.M.; KOTLYAR, A.B.; ALBRACHT, S.P. Energy-induced structural changes in NADH:Q oxireductase of the mitochondrial respiratory chain. **Biochim Biophys Acta**, 1186: 163-71, 1994.

KEGEL, K.B.; KIM, M.; SAPP, E.; McINTYRE, C.; CASTANO, J.G.; ARONIN, N.; DIFLIGIA, M. Huntington expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. **J Neurosci**, 20: 7268-78, 2000.

KERR, J.F.; WYLLIE, A.H.; CURRIE, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. **Br J Cancer**, 26: 239-57, 1972.

KIRINO, T. Delayed neuronal death in the gerbil hippocampus following ischemia. **Brain Res**, 239: 57-69, 1982.

KITAGAWA, K.; MATSUMOTO, M.; TSUJIMOTO, Y.; OHTSUKI, T.; KUWABARA, K.; MATSUSHITA, K.; YANG, G.; TANABE, H.; MARTINOU, J.C.; HORI, M.; YANAGIHARA, T. Amelioration of hippocampal neuronal damage after global ischemia by neuronal overexpression of Bcl-2 in transgenic mice. **Stroke**, 30: 2616-2621, 1998.

KLIONSKY, D.J.; EMR, S.D. Autophagy as a regulated pathway of cellular degradation. **Science**, 290: 1717-21, 2000.

KOGURE, K.; KATO, H. Neurochemistry of Stroke. In: H. J. M. BARNNET, E. M. STEIN; J. P. MOHR. **Stroke: Pathophysiology, diagnosis and management**. New York: Churchill Livingstone, 1993. p. 69-99.

KOWALTOWSKI, A.J.; CASTILHO, R.F.; GRIJALBA, M.T.; BECHARA, E.J.; VERCESI, A.E. Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation. **J Biol Chem**, 271: 2929-34, 1996.

KOWALTOWSKI, A.J.; CASTILHO, R.F.; VERCESI, A.E. Mitochondrial permeability transition and oxidative stress. **FEBS Lett**, 495: 12-15, 2001.

KOWALTOWSKI, A.J.; COSSO, R.G.; CAMPOS, C.B.; FISKUM, G. Effect of Bcl-2 overexpression on mitochondrial structure and function. **J Biol Chem**, 277: 42802-7, 2002.

KOWALTOWSKI, A.J.; NETTO, L.E.S.; VERCESI, A.E. The thiol specific antioxidant enzyme prevents mitochondrial permeability transition: Evidence for the reactive oxygen species in this mechanism. **J Biol Chem**, 273: 12766-69, 1998.

KOWALTOWSKI, A.J.; VERCESI, A.E. Mitochondrial damage induced by conditions of oxidative stress. **Free Radic Biol Med**, 26: 463-71, 1999.

KOWALTOWSKI, A.J.; VERCESI, A.E. Reactive oxygen generation by mitochondria. In: LEMASTERS, J.J. e NIEMINEN, A.L. **Mitochondria in Pathogenesis.** New York: Kluwer Academic/Plenum Publisher, 2001. p. 281-94.

KOWALTOWSKI, A.J.; VERCESI, A.E.; FISKUM, G. Bcl-2 prevents mitochondrial permeability transition and cytochrome *c* release via maintenance of reduced pyridine nucleotides. **Cell Death Differ**, 7: 903-10, 2000.

KRISTAL, B.S.; STAATS, P.N.; SHESTOPALOV, A.I. Biochemical characterization of the mitochondrial permeability transition in isolated forebrain mitochondria. **Dev Neurosci**, 22: 376-83, 2000.

KROEMER, G.; DALLAPORTA, B.; RESCHE-RIGON, M. The mitochondrial death/life regulator in apoptosis and necrosis. **Annu Rev Physiol**, 60: 619-42, 1998.

LAFON-CAZAL, M.; PIETRI, S.; CULCASI, M.; BOCKAERT, J. NMDA-dependent superoxide production and neurotoxicity. **Nature**, 364: 535-7, 1993.

LANCELOT, E.; BEAL, M.F. Glutamate toxicity in chronic neurodegenerative disease. **Prog Brain Res**, 116: 331-47, 1998.

LEDLEY, F.D. Perspectives on methylmalonic acidemia resulting from molecular cloning of methylmalonyl CoA mutase. **BioEssays**, 12: 335-40, 1990.

LEHNERT, W.; SPERL, W.; SUORMALA, T.; BAUMGARTINER, C.R. Propionic acidemia: Clinical, biochemical and therapeutic aspects. Experience in 30 patients. **Eur J Pediatr**, 153: 68-80, 1994.

LEHNINGER, A.L.; VERCESI, A.; BABABUNMI, E.A. Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides. **Proc Natl Acad Sci USA**, 75: 1690-4, 1978.

LEHNINGER, A.L. Fosforilação oxidativa e fotofosforilação. In: LEHNINGER, A.L.; NELSON, D.L.; COX, M.M. **Princípios de Bioquímica**. São Paulo: Sarvier, 1997. p. 404-43.

LEIST, M.; JÄÄTELLÄ, M. Four deaths and a funeral: from caspases to alternative mechanisms. **Nat Rev Mol Cell Biol**, 2: 589-98, 2001.

LINN, F.; PASCHEN, W.; OPHOFF, B.G.; HOSSMANN, K.A. Mitochondrial respiration during recirculation after prolonged ischemia in cat brain. **Exp Neurol**, 96: 321-33, 1987.

LIPTON, P. Ischemic cell death in brain neurons. **Phisiological Reviews**, 79: 1431-1568, 1999.

LIPTON, S.A.; ROSENBERG, P.A. Excitatory amino acids a final common pathway for neurologic disorders. **N Engl J Med**, 330: 613-22, 1994.

LIU, X.; KIM, C.N.; YANG, J.; JEMMERSON, R.; WANG, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. **Cell**, 86: 147-57, 1996.

LJUNGGREN, B.; SCHUTZ, H.; SIESJO, B.K. Changes in energy state and acid-base parameters of the rat brain during complete compression ischemia. **Brain Res**, 73: 277-89, 1974.

MCLAUGHLIN, B.A.; NELSON, D.; SILVER, I.A.; ERESCINSKA, M.; CHESSELET, M.F. Methylmalonate toxicity in primary neuronal cultures. **Neuroscience**, 86: 279-90, 1998.

MARCHETTI, P.; HIRSCH, T.; ZAMZAMI, N.; CASTEDO, M.; DECAUDIN, D.; SUSIN, S.A.; MASSE, B.; KROEMER, G. Mitochondrial permeability transition triggers lymphocyte apoptosis. **J Immunol**, 157: 4830-6, 1996.

MARKS, D.B.; MARKS, A.D.; SMITH, C.M. **Basic Medical Biochemistry: a clinical approach.** 1996.

MARTINOU, J.C.; FRANKOWSKI, H.; MISSOTTEN, M.; MARTINOU, I.; POTIER, L.; DUBOIS-DAUPHIN, M. Bcl-2 and neuronal selection during development of the nervous system. **J Physiol** (Paris), 88: 209-11, 1994.

MARTINOU, J.C.; GREEN, D.R. Breaking the mitochondrial barrier. **Nature Rev Mol Cell Biol**, 2: 63-7, 2001.

MATSUI, S. M.; MAHONEY, M. J.; ROSENBERG, L. E. The natural history of the inherited methylmalonic acidemias. **New Eng J Med**, 308: 857-61, 1983.

MATSUMOTO, S.; FRIBERG, H.; FERRAND-DRAKE, M.; WIELOCH, T. Blockade of the mitochondria permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion. **J Cereb Blood Flow Metab,** 19: 736-41, 1999.

MATTSON, M.P. Apoptosis in neurodegenerative disorders. **Nat Rev Mol Cell Biol**, 1: 120-9, 2000.

MATTSON, M.P.; CULMSEE, C.; YU, Z.F. Apoptotic and antiapoptotic mechanisms in stroke. **Cell Tissue Res**, 301: 173-87, 2000.

MELDRUM, B.; GARTHWAITE, J. Excitatory amino acid neurotoxicity and neurodegenerative disease. **Trends Pharmacol Sci**, 11: 379-87, 1990.

MERAD-SAIDOUNE, M.; BOITIER, E.; NICOLE, A.; MARSAC, C.; MARTINOU, J.C.; SOLA, B.; SINET, P.M.; CEBALLOS-PICOT, L. Overproduction of Cu/Zn-superoxide dismutase or Bcl-2 prevents the brain mitochondrial respiratory dysfunction induced by glutathione depletion. **Exp Neurol**, 158: 428-36, 1999.

MURPHY, A.N.; BREDESEN, D.E.; CORTOPASSI, G.; WANG, E.; FISKUM, G. Bcl-2 potentiates the maximal calcium uptake capacity of neuronal cell mitochondria. **Proc Natl Acad Sci USA**, 93: 9893-8, 1996.

NAGAHIRO, S.; UNO, M.; SATO, K.; GOTO, S.; MORIOKA, M.; UCHINO, Pathophysiology and treatment of cerebral ischemia. **J Med Invest**, 45: 57-70, 1998.

NAKANISHI, S.; MASU, M. Molecular diversity and functions of glutamate receptors. **Annu Rev Biphys Biomol Struct**, 23: 319-48, 1994.

NAKAO, N.; GRASBON-FRODL, E.M.; WIDNER, H.; BRUNDIN, P. Antioxidant treatment protects striatal neurons against excitotoxic insults. **Neuroscience**, 73: 185-200, 1996.

NICHOLLS, D.G.; BUDD, S.L. Mitochondria and neuronal survival. **Physiol Rev**, 80: 315-60, 2000.

NICOLLI, A.; BASSO, E.; PETRONILLI, V.; WENER, R.M.; BERNARDI, P. Interactions of cyclophilin with the mitochondrial inner membrane and regulation permeability transition pore, a cyclopsporin A-sensitive channel. **J Biol Chem**, 271: 2185-92, 1996.

NICOTERA, P.; LIPTON, S.A. Excitotoxins in neuronal apoptosis and necrosis. **J Cereb Blood Flow Metab**, 19: 583-91, 1999.

NISHIZUKA, Y.; HAYAISHI, O. Studies on the biosynthesis of nicotinamide adenine dinucleotide. **J Biol Chem**, 238: 3369-73, 1963.

NORDSTROM, C.H.; SIESJO, B.K. Effects of phenobarbital in cerebral ischemia. Part I: cerebral energy metabolism during pronounced incomplete ischemia. **Stroke**, 9: 327-35, 1978.

OHSUMI, Y. Molecular dissection of autophagy: two ubiquitin-like systems. **Nat Rev Mol Cell Biol**, 2: 211-6, 2001.

OKUN, J.G.; HORSTER, F.; FARKAS, L.M.; FEYH, P.; HINZ, A.; SAUER, S.; HOFFMANN, G.F.; UNSICKER, K.; MAYATEPEK, E.; KOLKER, S. Neurodegeneration in methylmalonic aciduria involves inhibition of complex II and the tricarboxylic acid cycle, and synergistically acting excitotoxicity. **J Biol Chem**, 277: 14674-80, 2002.

ORRENIUS, S.; ZHIVOTOVSKY, B.; NICOTERA, P. Regulation of cell death: the calcium-apoptosis link. **Nat Rev Mol Cell Biol**, 4: 552-65, 2003.

PELLEGRINI-GIAMPIETRO, D.E.; PERUGINELLI, F.; MELI, E.; COZZI, A.; ALBANI-TORREGROSSA, S.; PELLICCIARI, R.; MORONI, F. Protection with metabotropic glutamate 1 receptor antagonists in models of ischemic neuronal death: time-course and mechanisms. **Neuropharmacology**, 38:1607-19, 1999.

PETTENUZZO, L.F. **Efeito da administração crônica pós-natal dos ácidos propiônico e metilmalônico sobre o comportamento de ratos no labirinto aquático de Morris (Water Maze) e sobre alguns parâmetros bioquímicos de estresse oxidativo no hipocampo**. Porto Alegre, 2001. (Tese - Mestrado - Universidade Federal do Rio Grande do Sul).

PETTMANN, B.; HENDERSON, C.E. Neuronal cell death. **Neuron**, 20: 633-47, 1998.

PULSINELLI, W. Pathophysiology of acute ischaemic stroke. **Lancet**, 339: 533-6, 1992.

PULSINELLI, W.A. Selective neuronal vulnerability and infarction in cerebrovascular disease. In: WELCH, K.M.A.; REIS, D.J.; CAPLAN, L.R.; SIESJÖ, B.K.; WEIR, B. **Primer on cerebrovascular diseases**. San Diego: Academic Press, 1997.

PULSINELLI, W.A.; DUFFY, T.E. Regional energy balance in rat brain after transient forebrain ischemia. **J Neurochem**, 40: 1500-3, 1987.

QIN, Z.H.; WANG, Y.; CHASE, T.N. Stimulation of *N*-methyl-D-aspartate receptors induces apoptosis in rat brain. **Brain Res**, 725:166-76, 1996.

RAVAGNAN, L.; ROUMIER, T.; KROEMER, G. Mitochondria, the killer organelles and their weapons. **J Cell Physiol**, 192: 131-37, 2002.

REYNOLDS, I.J. Intracellular calcium and magnesium: critical determinants of excitotoxicity? **Prog Brain Res**, 116: 225-43, 1998.

ROE, C.R.; HOPPEL, C.L.; STACEY, T.E.; CHALMERS, R.A.; TRACEY, B.M.; MILLINGTON, D.S. Metabolic response to carnitine in methylmalonic aciduria. An effective strategy for elimination of propionyl groups. **Arch Dis Child**, 58: 916-20, 1983.

SCHENDEL, S.L.; XIE, Z.; MONTAL, M.O.; MATSUYAMA, S.; MONTAL, M.; REED, J.C. Channel formation by antiapoptotic protein Bcl-2. **Proc Natl Acad Sci USA**, 94: 5113-8, 1997.

SCHMIDT-KASTNER, R.; FREUND, T.F. Selective vulnerability of the hippocampus in brain ischemia. **Neuroscience**, 40: 599-636, 1991.

SCRIVER, C.R.; BEAUDET, A.L.; SLY, W.S.; VALLE, D. **The metabolic and molecular bases of inherited disease**. New York: McGraw-Hill, 2001.

SHIERLE, G.S.; LEIST, M.; MARTINOU, J.C.; WIDNER, H.; NICOTERA, P.; BRUNDIN, P. Differential effects of Bcl-2 overexpression on fibre outgrowth and survival of embryonic dopaminergic neurons in intracerebral transplants. **J Neurosci**, 11: 3073-81, 1999.

SHIMIZU, S.; EGUCHI, Y.; KAMIIKE, W.; FUNAHASHI, Y.; MIGNON, A.; LACRONIQUE, V.; MATSUDA, H.; TSUJIMOTO, T. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. **Proc Natl Acad Sci USA**, 95: 1455-59, 1998.

SIEMKOWICZ, E. Brain uptake of mannitol and sucrose after cerebral ischemia: effect of hyperglycemia. **Acta Physiol Scand**, 112: 359-62, 1981.

SIESJÖ, B.K.; JOHAMNSSON, H.; LJUNGGREN, B.; NORBERG, K. Brain dysfunction in cerebral hypoxia and ischemia. **Res Publ Assoc Res Nerv Ment Dis**, 53: 75-112, 1974.

SIESJÖ, B.K.; SIESJÖ, P. Mechanisms of secondary brain injury. **Eur J Anaesthesiol**, 13: 247-68, 1996.

SKULACHEV, V.P. Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. **Q Rev Biophys**, 29: 169-202, 1996.

SORRENTINO, V.; VOLPE, P. Ryanodine receptors: how many, where and why? **Trends Pharmacol Sci**, 14: 98-103, 1993.

SOUSA, S.C.; MACIEL, E.N.; VERCESI, A.E.; CASTILHO, R.F. Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. **FEBS Lett,** 543: 179-83, 2003.

STADTMAN, E.R. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. **Free Radical Biol Med**, 9: 315-25, 1990.

STARKOV, A.A.; POLSTER, B.M.; FISKUM, G. Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax. **J Neurochem**, 83: 220-8, 2002.

SWEETMAN, L.; WILLIAMS, J.C. Branched chain organic acidurias. In: SCRIVER, C. R.; BEAUDET, A. L.; SLY, W. S.; VALLE, D. **The Metabolic and Molecular Basis of Inherited Disease**. New York: McGraw-Hill, 2001. p. 2155-7.

THORNBERRY, N.A.; LAZEBNIK, Y. Caspases: enemies within. **Science**, 281: 1312-6, 1998.

TSUJIMOTO, M.; YIP, Y.K.; VILCEK, J. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. **Proc Natl Acad Sci USA**, 82: 7626-30, 1985.

TURRENS, J.F. Superoxide production by mitochondrial respiratory chain. **Biosci Rep**, 17: 3-8, 1997.

TURRENS, J.F.; BOVERIS, A. Generation of the superoxide anion by NADH dehydrogenase of bovine heart mitochondria. **Biochem J**, 191: 421-7, 1980.

TYMIANSKI, M.; CHARLTON, M.P.; CARLEN, P.L.; TATOR, C.H. Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. **J Neurosci**, 13: 2085-104, 1993.

UCHINO, H.; ELMER, E.; UCHINO, K.; LI, P.A.; HE, Q.P.; SMITH, M.L.; SIESJÖ, B.K. Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. **Brain Res**, 812: 216-26, 1998.

VALLE, V.G.R.; FAGIAN, M.M.; PARENTONI, L.S.; MEINICKE, A.R.; VERCESI, A.E. The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants. **Arch Biochem Biphys**, 307: 1-7, 1993.

VAUX, D.L.; CORY, S.; ADAMS, J.M. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. **Nature**, 335: 440-2, 1988.

VERCESI, A.E. The participation of NADP, the transmembrane potential and the energy-linked NAD(P) transhydrogenase in the process of Ca^{2+} efflux from rat liver mitochondria. **Arch Biochem Biophys**, 252: 171-8, 1987.

VERCESI, A.E.; KOWALTOWSKI, A.J.; GRIJALBA, M.T.; MEININKE, A.R.; CASTILHO, R.F. The role of reactive oxygen species in mitochondrial permeability transition. **Biosci Reports**, 17: 43-52, 1997.

VOET, D. Transporte de elétrons. In: **Fundamentos de Bioquímica**. Porto Alegre: Artmed, 2002. p. 497-507.

WAJNER, M.; DUTRA, J.C.; CARDOSO, S.E.; WANNMACHER, C.M.D.; MOTTA, ER. Effect of methylmalonate on *in vitro* lactate release and carbon dioxide production by brain of suckling rats. **J Inherit Metab Dis**, 15: 92-6, 1992.

WANNMACHER, C.D.; DUTRA, R.D. Metabolismo de Lipídeos. In: **Bioquímica Fundamental**. Porto Alegre, 1988. p. 261-75.

WESTBROOK, G.L. Glutamate receptor update. **Curr Opin Neurobiol**, 4: 337-46, 1994.

WYLLIE, A.H.; KERR, J.F.; CURRIE, A.R. Cell death: the significance of apoptosis. **Int Rev Cytol**, 68: 251-306, 1980.

WOLF, B.B.; GREEN, D.R Suicidal tendencies: apoptotic cell death by caspase family proteinases. **J Biol Chem**, 274: 20049-52, 1999.

YANG, J.C.; KAHAN, A.; CORTOPASSI, G. Bcl-2 does not inhibit the permeability transition pore in mouse liver mitochondria. **Toxicology**, 151: 63-72, 2000.

YANG, L.; MATTHEWS, R.T.; SCHULZ, J.B.; KLOCKGETHER, T.; LIAO, A.W.; MARTINOU, J.C.; PENNEY Jr, J.B.; HYMAN, B.T.; BEAL, M.F. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenuated in mice overexpressing Bcl-2. **J Neurosci**, 18: 1845-52, 1998.

ZAGO, E.B.; CASTILHO, R.F.; VERCESI, A.E. The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. **FEBS Lett**, 478: 29-33, 2000.

ZAMZAMI, N.; MARZO, L.; SUSIN, S.A.; BRENNER, C.; LAROCHETTE, N.; MARCHETTI, P.; REED, J.; KOFLER, R.; KROEMER, G. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. **Oncogene**, 16: 1055-63, 1998.

ZIMMERMANN, K.C.; BONZON, C.; GREEN, D.R. The machinery of programmed cell death. **Pharmacol Ther**, 92: 57-70, 2001.

ZORATTI, M.; SZABÒ, L. The mitochondrial permeability transition. **Biochem Biophys Acta**, 1241: 139-76, 1995.

6- APÊNDICE

ANEXO – CURRICULUM VITAE

1- INFORMAÇÕES PESSOAIS

1.1. Nome: Evelise Neves Maciel

1.2. Filiação: Elisabeti Neves Maciel

Manoel Antônio Maciel

1.3. Naturalidade: São Gabriel, RS, Brasil

1.4. Data de Nascimento: 13/10/1973.

2- ESCOLARIDADE

2.1. 1980-1987 – 1.° Grau -

Colégio Marista Santo Ângelo, Santo Ângelo, RS.

2.2. 1988-1990 – 2.° Grau -

Colégio Marista Santo Ângelo, Santo Ângelo, RS.

2.3. 1994-1997 - Graduação em Ciências Biológicas - Universidade Federal de Santa Maria (UFSM), Santa Maria, RS.

2.4. 1999-2000 - Mestrado em Ciências Biológicas - Bioquímica - Departamento de Bioquímica

Universidade Fed. do Rio Grande do Sul (UFRGS), Porto Alegre, RS

Orientador: Prof. Dr. João Batista Teixeira da Rocha

3 - PUBLICAÇÕES EM REVISTAS ESPECIALIZADAS

3.1. **MACIEL, E.N.**; BOLZAN, R.C.; BRAGA, A.L.; ROCHA, J.B.T. Diphenyl diselenide and diphenyl diteluride affect δ-aminolevulinate dehydratase from liver, kidney and brain of mice. **J Biochem Mol Toxicol**, 14: 310-319, 2000.

3.2. **MACIEL, E.N.**; VERCESI; A.E.; CASTILHO, R.F. Oxidative stress in Ca^{2+} -induced membrane permeability transition in brain mitochondria. **J Neurochem**, 79: 1237-1245, 2001.

3.3. **MACIEL, E.N.**; FLORES, E.M.M.; ROCHA, J.B.T.; FOLMER, V. Deposition of diphenyl diselenide in liver, kidney and brain of mice. **Bull Environ Contam Toxicol**, 70: 470-476, 2003.

3.4. SOUSA, S.C.; **MACIEL, E.N.**; VERCESI, A.E.; CASTILHO, R.F. Ca^{2+} -induced oxidative stress in brain mitochondria treated with respiratory chain inhibitor rotenone. **FEBS Lett**, 543: 179-183, 2003.

3.5. **MACIEL, E.N.**; SCHIERLE, G.S.K.; HANSSON, O.; BRUNDIN, P.; CASTILHO, R.F. Cyclosporin A and Bcl-2 do not inhibit quinolinic acid-induced striatal excitotoxicity in rodents. Implications for the occurence of mitochondrial permeability transition *in vivo*. **Exp Neurol**, 183: 430-437, 2003.

3.6. **MACIEL, E.N.**; KOWALTOWSKI, A.J.; SCHWALM, F.D.; RODRIGUES, J.M.; SOUZA, D.O.; VERCESI, A.E.; WAJNER, M.; CASTILHO, R.F. Mitochondrial permeability transition in neuronal damage promoted by Ca^{2+} and respiratory chain complex II inhibition. **J Neurochem**, versão re-submetida, 2004.